Naveen Kumar Arora *Editor*

Plant Microbes Symbiosis: Applied Facets

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Preface

Plant associated microorganisms (PAMs) play a number of very important roles to better the life of their host plant. It is believed that the origin of plant-microbe interactions is as old as the arrival of plants on land, and both the mutual partners selected each other after a long evolutionary process. These useful microbes provide the plants with nutrients, battle pathogens, combat abiotic stresses, and can play several other roles in the plant ecosphere. Plant-microbe interactions in tandem can perform acts such as reclamation of barren soils, degradation of recalcitrant pollutants, and removal of dangerous compounds from the ecosystems.

Injudicious use of chemicals in agriculture and industries has led to major environmental and health related issues for humans and other organisms inhabiting the planet. These chemicals are accumulating in the food chain and ecosystems. In fact, so many of them reach into the food products which we consume for our sustenance. The indiscriminate use of chemicals is in fact responsible for the reduced fertility of soils by killing several important and useful PAMs. In case of microorganisms we even do not know what we have lost over the years. But one thing is certain that in barren soils the population of useful plant growth promotory (PGP) microbes is very low or even more alarmingly they may altogether be absent from such soils. Hence there is an urgent need to re-introduce these beneficial microbes in the soil along with their compatible plants. The book discusses the various applications of PGP microbes and the importance and utility of plant-microbe interactions in sorting out the issues related to soil degradation and pollution.

The volume is in the form of 19 chapters contributed by experts, from around the globe, involved in research on applied utility of plant-microbe interactions. The tome discusses in great detail the present scenario of utilizing the PGP microbes for enhancement of crop yields by providing nutrients through biocycling or mobilization or fixation, fighting phytopathogens and other stresses such as salinity, drought, pH, etc. An integrated management of nutrients, diseases, and stresses can be achieved by application of diverse PGP microbes in combination. However, to provide these benefits to the plant the PGP microbes have to be augmented in the soil, in particular in the habitats where they are deficient in numbers. This can only be achieved by uplifting the quality of current bioinoculants by evaluating the possible bottlenecks and taking cue from latest research for the development of novel futuristic bioinoculants. Utilization of advanced biotechnological tools such as

genomics, proteomics, and genetic engineering can go a long way in achieving the targets. Bt crops are already proving to be a big success story for the case. Exploitation of plant-microbe symbiosis for reclamation of barren or polluted lands, degradation of pollutants, and combating the impact of climate change are the future directions to be explored. The book focuses on these very issues which will be extremely important for fulfilling the food security aspirations of swelling population on reducing arable land in a sustainable manner.

Researchers involved in the field of bioinoculant technology, PGPRs, plant-microbe interactions, rhizosphere biology, bioremediation, biotic and abiotic stress management for crop production, and related areas will find the compilation extremely useful. The researchers will not only get a comprehensive insight on what is happening in this field around the globe but will also be able to know about the future directions of research. The book will be very useful for faculty and graduate and post-graduate students of life sciences, specifically those pursuing career in agriculture microbiology or biotechnology. The book discusses in detail how the plant-microbe symbiosis have been utilized so far and how we can utilize this wonderful relationship in future so as to use fewer chemicals and ensuring for future generations safe and sustainable agri-ecosystems.

I would like to thank all the contributors for providing great knowledge on diverse aspects of the subject. The applications of plant-microbe associations have been comprehensively covered in the tome due to the vast expertize of the authors, and I can say that it is due to their effort that the book has become unique in itself. I thank Dr. Mamta Kapila, Senior Editor, Springer (India), for her continuous support and pursuance. It is because of her that the project could stick to the time frame and see the light of day. My special thanks to Prof. R. C. Sobti, Vice Chancellor, BBA University, Lucknow, for his incessant support and encouragement. I would like to thank the team of my research scholars including Sakshi Tewari, Jitendra Mishra, Rachna Singh, Maya, Shweta and Jay Prakash for helping in compilation of the manuscript. Most importantly, I have to acknowledge the support and care of my wife, Preeti Arora, for looking after me and my kids, Pranay and Nav, who by their presence make all the tensions and fatigue go burst.

I hope that the readers will find the book very beneficial. For any further suggestions or corrections please get back to yours truly.

Lucknow, Uttar Pradesh, India Naveen Kumar Arora

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About the Editor

Dr. Naveen Kumar Arora, Ph.D. Microbiology, Associate Professor and Head, Department of Environmental Microbiology, Babasaheb Bhimrao Ambedkar University (A Central University), Lucknow, Uttar Pradesh, India, is a renowned researcher in the field of Environmental Microbiology and Biotechnology. His specific area of research is rhizosphere biology and PGPRs. He has 41 research papers published in premium international journals and several articles published in magazines and dailies. He is an editor of the book *Plant Microbe Symbiosis: Fundamentals and Advances*, published by Springer. He is a member of several national and international societies and reviewer of several international journals. He has delivered lectures in conferences and seminars around the globe. He has a long-standing interest in teaching at the PG level and is involved in taking courses in bacteriology, microbial physiology, environmental microbiology, agriculture microbiology, and industrial microbiology. He has been an advisor to 57 post-graduate and 3 doctoral students. Recently, he was awarded for excellence in research by the Honorable Governor of Uttar Pradesh. Although an academician and researcher by profession, he has a huge obsession for the wildlife and its conservation and has authored a book, *Splendid Wilds*. He has a dedicated website www.naveenarora.co.in for the cause of wildlife and environment conservation.

Rhizosphere Bacteria for Crop Production and Improvement of Stress Tolerance: Mechanisms of Action, Applications, and Future Prospects

1

Sajid Mahmood Nadeem, Muhammad Naveed, Maqshoof Ahmad, and Zahir Ahmad Zahir

Abstract

Rhizosphere bacteria associated with plant roots can enhance crop productivity through a number of direct and indirect mechanisms. These beneficial bacteria attracted the scientists around the globe due to their significant contribution to mitigate adverse effects of environmental stresses on plants. These plant growth-promoting rhizobacteria (PGPR) have the potential to improve crop production under stress conditions solely and/or in combination with other microbes. The use of PGPR as co-inoculants with symbiotic bacteria is a potential biotechnological approach to promote nodulation for improving crop biomass and soil health. Multi-strain bacterial consortia are also proved useful for enhancing plant growth and development particularly in conditions where single inoculation was not so effective. The objectives of present review are to highlight the basic mechanisms used by such bacteria in general and the applied aspects of these bacteria for improving plant stress tolerance and ultimately crop productivity particularly. Certain examples highlighting their significant role for enhancing plant growth under biotic and abiotic stress conditions have been reviewed. The role of PGPR for improving nodulation when used with nitrogen-fixing bacteria has been discussed. The potential of genetically engineered rhizobacteria that possess the required trait necessary under certain environmental conditions has also been evaluated. The areas that need further research and future perspectives of this technology have been discussed in detail.

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Introduction

Although plants are well adapted to a number of environmental conditions, certain biotic and abiotic stresses cause significant reduction in plant growth and yield. When plants are subjected to environmental stresses, a number of plant processes are affected that ultimately lead to improper growth and development. Under stress, plants reduce their vegetative growth to conserve and redistribute resources that decrease their chances of survival under severe conditions (Skirycz and Inze 2010). These stresses affect the plant growth and development in one way or the other. For example, in water-limited conditions, reduction in initial plant growth occurs that results in plant leaves with fewer and smaller cells (Aguirrezabal et al. 2006; Skirycz et al. 2010). Saline conditions affect the plant growth by a number of negative impacts including ion toxicity, osmotic effect, and specific ion effect (Munns 1993; Ashraf 1994; Sosa et al. 2005; Conde et al. 2011; Habib and Ashraf 2014). The presence of heavy metals and other contaminants also reduces the crop productivity by disturbing various plant processes (Aydinalp and Marinova 2009; Yadav 2010).

In the context of climate change and mounting population pressure, increasing plant tolerance against these stresses is a dire need. A number of techniques have been adopted to understand the mechanisms and providing tools to enhance plant tolerance against environment stresses. Some of these techniques are based on the use of chemicals that may be toxic for environment. For example, there is environmental concern about the application of aminoethoxyvinylglycine (a chemical inhibitor of ethylene) for protecting the plant from stress-induced impact of ethylene. Similarly, there are also concerns about the use of fertilizers, herbicides, fungicides, and pesticides, which provide nutrition as well as protect the plant from pest and diseases.

One of the alternatives and emerging technologies to solve this problem is the use of naturally occurring PGPR. There are a number of reports that show the effectiveness of PGPR for enhancing plant growth and development (Zahir et al. 2004; Paul and Nair 2008; Glick 2012). This environment-friendly population is equally effective for promoting crop productivity under normal and stress conditions (Zahir et al. 2004; Nadeem et al. 2010b; Saharan and Nehra 2011). There are a number of reports revealing the mechanism of growth promotion by PGPR that may be direct or indirect (Nadeem et al. 2010b; Glick 2012; Ahemad and Kibret 2014).

The use of this environment-friendly approach could be one of the alternatives to minimize the use of chemicals, which cause a number of negative impacts on human health directly and indirectly. The use of this technology could also be useful for improving yield of agricultural crops that otherwise looks difficult due to the limited availability of agricultural inputs as well as adverse environmental conditions. The present review highlights the significant impact of PGPR on plant growth and development under stress environment. Some important aspects of PGPR that make it a useful approach for sustainable agriculture have been discussed in detail.

Rhizosphere: A Nutrient-Rich Niche for Beneficial Bacteria

The zone surrounding the plant roots in which complex relations exist between plant, microorganisms, and the soil is called rhizosphere. According to Hiltner (1904), it is a region of soil around the plant root where maximum microbial activity is present compared to surrounding soil. Plant root exudates are a source of food for microbes and these exudates support the growth and development of microbial population. In view of Hiltner, not only the bacteria present in the rhizosphere are attracted by the root exudates but there are also "uninvited guests" that use these exudates for their survival (Hartmann et al. 2008). The chemical changes occurring at soil–root interface have marked influence on plant microbial interactions as well as availability and acquisition of nutrients. The rhizosphere soil is a favorable habitat for microbial growth which exerts a potential impact on plant health as well

as soil fertility (Antoun and Prevost 2005; Podile and Kishore 2006).

In the rhizosphere, a number of important and intensive interactions occur among microorganisms, plant, as well as soil microfauna (Antoun and Prevost 2005). As diverse interactions between organisms take place in this region, it is also described as "microbial hot spot" (Whipps 2001). These interactions are based on complex exchanges between roots and microorganisms, and the beneficial, detrimental, and neutral nature of these relationships is all regulated by complex molecular signaling (Dardanelli et al. 2010). The beneficial interactions can significantly influence plant growth and development, and therefore have a positive influence on crop production.

Root exudates contain adequate amount of carbon and energy that is readily available for microbial growth and development and the physiology of microbial cell (Somers et al. 2004). These exudates consist of a number of organic compounds, auxins, sugars, vitamins, and flavonoids (Neumann and Romheld 2001; Somers et al. 2004; Dardanelli et al. 2009; Raaijmakers et al. 2009). Due to the presence of a variety of compounds in the root exudates, microorganisms like PGPR are present in high concentration around the roots and utilize these compounds as food to promote their growth and development (Whipps 1990; Glick et al. 1999).

The length of the rhizosphere soil may vary and range from less than 1 mm to several millimeters depending upon the presence of root exudates (Darrah 1993; Hubel and Beck 1993; Bringhurst et al. 2001). The microbial population in the rhizosphere is different from surrounding environment owing to the presence of large amounts of nutrients released by plant roots (Burdman et al. 2000). According to an estimate, the number of microorganisms in the rhizosphere is 19–32 times higher than surrounding bulk soil, i.e., free of roots (Bodelier et al. 1997). Due to the presence of exudates in this area, most microorganisms occur within 50 μm of root surface. About 50–100 mg of exudates are released from per gram of root that is enough to support 2×10^{10} bacterial cells (Foster 1983). Although a large number of microbial population are present in the rhizosphere, only 7–15 % of the total root surface is generally occupied by microbial cells (Foster 1983; Pinton et al. 2001).

Due to the nutrient-rich environment compared to surrounding bulk soil, the competition among microbial population for nutrients is very high in this environment (Berg et al. 2005). The rhizosphere microorganisms having the ability to grow rapidly by degrading substances like chitin, cellulose, and root and seed exudates control the rhizosphere competition (Baker 1991), and being the most abundant microorganisms in the rhizosphere, bacteria are highly competitive and aggressively colonize the plant roots (Antoun and Prevost 2005; Bouizgarne 2013).

Plant Growth-Promoting Rhizobacteria: An Overview

The term plant-growth promoting rhizobacteria (PGPR) was first used by Kloepper and Schroth (1978) to describe the bacteria that colonize the plant roots. These microorganisms generally exist more or less near the roots due to the presence of root exudates that are a source of food for their growth (Whipps 1990), and most of these microbes depend on root exudates for their survival (Glick et al. 1998; Khalid et al. 2006). Some of these PGPR belong to important genera including *Serratia*, *Arthrobacter*, *Azospirillum*, *Pseudomonas*, *Burkholderia*, *Enterobacter*, *Bacillus*, *Azoarcus*, *Paenibacillus*, *Klebsiella*, *Erwinia*, *Beijerinckia*, *Flavobacterium*, and *Gluconacetobacter* (Okon and Labandera-Gonzalez 1994; Glick 1995; Podile and Kishore 2006; Dardanelli et al. 2009).

Some researchers have described PGPR on the basis of their growth-promoting characteristics. For example, Bashan and Holguin (1998) categorized the PGPR into two classes, i.e., biocontrol PGPB (plant growth-promoting bacteria) and PGPB. Khan (2005) on the basis of the relationship of rhizobacteria with plants divided them into two groups, i.e., symbiotic rhizobacteria and free-living rhizobacteria. However, Gray and Smith (2005) classified PGPR as extracellular and intracellular. According to their view,

extracellular PGPR exist in the rhizosphere, on the rhizoplane, or in the spaces between cells of the root cortex, while intracellular PGPR exist inside the root cells. Sayyed et al. (2010) termed beneficial microorganisms as yield-increasing bacteria (YIB) or PGPR. Burr and Caesar (1984) reported these bacteria as plant health-promoting rhizobacteria (PHPR) and nodule-promoting rhizobacteria that are present in the rhizosphere. In view of Vessey (2003), PGPR are bacteria which flourish in the rhizosphere and may grow in, on, or around plant tissues and enhance growth and development. Gray and Smith (2005) mentioned the gradient of root proximity among rhizobacteria, including bacteria living near roots, bacteria in the rhizoplane, bacteria within the root tissues, and bacteria that reside inside the cell in specialized structures called nodule.

There are a number of ways through which PGPR are helpful for promoting plant growth and development. According to Glick (1995), PGPR facilitate plant growth by three different ways that include the synthesis of compounds for plant uptake, facilitating the nutrient uptake, and disease prevention (Garcia de Salamone et al. 2001; Raj et al. 2003; Guo et al. 2004). PGPR use various mechanisms which may take place simultaneously or sequentially at different plant growth stages. These mechanisms are divided into two major categories, i.e., direct and indirect growth-promoting mechanisms (Scher and Baker 1982; Shanahan et al. 1992; Glick 1995; Boddey and Dobereiner 1995; Glick et al. 1995; Flaishman et al. 1996). Nitrogen fixation, phosphate solubilization, and phytohormone and siderophore production are some examples of direct growth promotion by the PGPR (Kloepper et al. 1989; Glick et al. 1995). PGPR also play an important role indirectly in plant growth promotion by inhibiting the growth of plant pathogens (Glick and Bashan 1997; Persello-Cartieaux et al. 2003). This can be achieved by the production of antibiotic and antifungal metabolites, lytic enzymes, and hydrogen cyanide, inducing systemic resistance and also by reducing the availability of certain nutrients such as iron required by the pathogen for its growth (Kloepper and Mariano 2000; Antoun and Kloepper 2001; Van Loon 2007; Labuschagne et al. 2010).

In addition to these general mechanisms of growth promotion, PGPR also protect the plant from the deleterious effects of various stresses. Stress conditions affect not only plant growth but are also harmful for the microbial community. However, under stress conditions, certain PGPR maintain their growth by using particular mechanisms. For example, *Pseudomonas* sp. has the ability to withstand drought stress environment by the production of exopolysaccharides (EPS) (Sandhya et al. 2009). PGPR also enhance plant growth under stress conditions like salinity, drought, heavy metals, flooding, temperature, and petrochemicals like diesel (Saleem et al. 2007; Nadeem et al. 2010b). Under stress conditions PGPR work through particular mechanisms like lowering of stress-induced ethylene, production of EPS, regulating nutrient uptake, enhancing the activity of antioxidant enzymes, and suppressing the growth of pathogens (Glick et al. 2007; Sandhya et al. 2009; Belimov et al. 2009a; Nadeem et al. 2010a; Saharan and Nehra 2011).

Rhizosphere Bacteria for Plant Biomass Production

For maintaining soil function, the presence of microorganisms is very essential because they play critical role in decomposition, nutrient cycling, toxin removal, and improving soil structure (van Elsas and Trevors 1997). Their use for improving nutrient status of soil is very essential and important for agricultural practices (Freitas et al. 2007). There are several reports showing the remarkable role of these microbes for improving plant growth and yield of a number of crops (Kloepper et al. 1980; Biswas et al. 2000; Asghar et al. 2002; Zahir et al. 2004; Gray and Smith 2005; Figueiredo et al. 2008; Nadeem et al. 2010b).

It has been observed that certain PGPR strains have advantage to protect the plant from more than one stress. This growth promotion under different environments indicates that these strains

utilize more than one mechanism to promote growth in that particular environment. This might be due to their various growth-promoting traits like 1-aminocyclopropane-1-carboxylate (ACC) deaminase activity and EPS production, inducing systemic resistance (ISR) and better root colonization (Bakker et al. 2007; Sandhya et al. 2009; Zahir et al. 2009; Nadeem et al. 2010b). Some of the important aspects of PGPR that are useful for plant biomass have been reviewed and discussed in the following sections.

Biofertilizers

With increase in population, the demand for food is also increasing. No doubt, the use of modern technologies has increased crop production many times compared to old conventional approaches. However, this increase in crop yield is generally due to the use of excessive amount of fertilizers (i.e., the requirement of high-yielding varieties), pesticides, and other commercial growth-promoting substances. In addition to increase in crop yield, most of these substances also cause negative impact on environment. The excessive losses of chemical fertilizer, pesticides, and other waste products cause environmental and social problems throughout the world (Singh et al. 2011). Although certain physical and chemical methods are being adopted to solve such problems, it has been observed that it cannot be done effectively without applying microbial methods and techniques (Singh et al. 2011).

One of the environment-friendly techniques is the use of biofertilizers. A biofertilizer is a product that contains living microorganisms, which enhance plant growth and yield through direct or indirect mechanisms (Fuentes-Ramirez and Caballero-Mellado 2005). According to Vessey (2003), biofertilizer is a microbial product, which when applied colonizes the rhizosphere or plant interior and enhances the growth of plant by increasing the availability or supply of nutrients.

Biofertilizers are important components of integrated nutrient systems, and their use (containing beneficial microorganisms) instead of synthetic chemicals is known to improve plant growth and development and may help to sustain environmental health and soil productivity (O'Connel 1992; Wu et al. 2005). The mechanisms of biofertilizer for improving plant growth are not still understood clearly; however, some growth-promoting attributes have been mentioned in the literature, which include nitrogen fixation, phytohormone production, solubilization, and availability of nutrients, reducing stress impact by enzymes like ACC deaminase, chitinase, etc. (Vessey 2003; Glick 2012; Ahemad and Kibret 2014). Some of the selected examples of the effect of biofertilizers on different crops have been reviewed in the following sections.

Cereals

Cereals like wheat, maize, and rice are the main staple food for most of the world population. Due to an increase in population, their demand is also increasing. The extensive use of chemical fertilizers for increasing yield of such valuable crops results in a negative impact on environment. Moreover, these nonleguminous crops do not fix nitrogen, and therefore, application of nitrogen to these crops is another expensive input. However, the use of PGPR can improve the nitrogen uptake of nonleguminous crops as it was observed in maize (31 % N) and palm seedlings where inoculated plants showed 31 and 40 % more nitrogen, respectively, compared to uninoculated control (El-Komy et al. 1998; Amir et al. 2001). Similarly, inoculation of nitrogen-fixing and phosphate-solubilizing bacteria (PSB), in combination, proved more effective for enhancing nutrient uptake in various crops including sorghum, barley, and wheat (Alagawadi and Gaur 1992; Belimov et al. 1995; Galal 2003).

Yazdani et al. (2009) in a field experiment observed that application of manure as well as biofertilizer enhanced the yield of maize. They further concluded that application of PSB along with other PGPR strains reduced the application of phosphorus up to 50 % without any reduction of yield. They concluded that up to 50 % nitrogen fertilizer could be saved by inoculation with

Azospirillum and phosphobacteria. However, in earlier study conducted on millet, Chandrasekar et al. (2005) demonstrated that the effectiveness of biofertilizer was more when used with chemical fertilizers. The efficiency of biofertilizer also increased when used with other microbial populations. For example, the use of mycorrhizal fungi along with bacteria enhances the effectiveness of biofertilizers. Wu et al. (2005) developed two biofertilizers containing arbuscular mycorrhizal fungi (*Glomus mosseae* and *Glomus intraradices*) with or without bacterial strains (nitrogen fixer, *Azotobacter chroococcum*; PSB, *Bacillus megaterium*; and K solubilizer, *Bacillus mucilaginous*). Effect of biofertilizer was evaluated on soil particles and maize (*Zea mays*) growth. They also compared the efficiency of these biofertilizers with chemical and organic fertilizers. The results showed that the biofertilizer containing mycorrhizal fungi with three strains of rhizobacteria significantly enhanced the growth of maize. The application of biofertilizer not only improved the plant growth but also caused positive effect on soil properties. The organic matter and nitrogen contents of soil increased with the application of biofertilizer. It was also observed that half the amount of biofertilizer caused similar effect when compared with organic and chemical fertilizer.

Legumes

Legumes are well known for their ability to fix atmospheric nitrogen, which may be a good source of nitrogen for the succeeding crop. To fulfill the phosphorus requirements of plants, soluble phosphatic fertilizers are preferably used for crop production; however, the manufacturing cost of chemical fertilizers makes them economically unaffordable. Stamford et al. (2007) conducted a study to minimize this problem by using biofertilizers. Experiments were carried out on yam bean (*Pachyrhizus erosus*) in low-P available soil. Biofertilizer was prepared from rock phosphate and sulfur and then inoculated with *Acidithiobacillus*. An increase in soil acidity and available P was observed where biofertilizers were applied. This study indicates better performance of biofertilizer for enhancing growth.

Vermicompost, a biofertilizer that contains worms, beneficial bacteria, and a number of major and micronutrients, proves very useful for enhancing plant growth. Jeyabal and Kuppuswamy (2001) studied the response of vermicompost prepared from agricultural and industrial waste by using earthworms (*Eudrilus eugeniae*) along with fertilizer nitrogen and another biofertilizer prepared from *Azospirillum* and phosphobacteria on rice–legume system. The study showed that application of vermicompost, *Azospirillum*, and phosphobacteria biofertilizer increased rice yield compared to fertilizer nitrogen alone. The residual effect of these fertilizers was studied on the succeeding legume. The residual effect of N supplied through vermicompost and *Azospirillum* produced higher nodule number and nodule weight than fertilizer N alone. The yield was also higher in case of biofertilizer compared to nitrogen fertilizer. Similarly, Sinha et al. (2010) studied the efficacy of vermicompost against DAP (diammonium phosphate) and FYM (farm yard manure) on *Cicer* and *Pisum*. A significant improvement of growth parameters like root length, shoot length, number of pods, and number of nodules in both crops was observed compared to untreated control. The applications of biofertilizer also increased the population of nitrogen-fixing bacteria.

The application of biofertilizers has proved not only useful for enhancing growth and yield of grain legumes but also the biomass of fodder legume crops. *Galega orientalis* is a perennial leguminous fodder crop. Biofertilizer caused positive effect on the growth of this fodder crop (Arkhipchenko et al. 2005). Three biofertilizers were prepared from pig farm sludge and poultry dung from aerobic and poultry dung from anaerobic conditions and then checked for quality, nutrient content, and bacterial communities. *Bacillus* spp. represented maximum microbial population, i.e., 20–35 % of the viable heterotrophic microorganisms in the final product. The population of pathogenic fungi was suppressed in the final product which might be due to *Bacillus* sp. because of their ability to produce antibiotics to suppress the growth of phytopathogenic fungi (Foldes et al. 2000; Knox et al. 2000). Application

of these fertilizers to *G. orientalis* significantly increased the yield. It was also observed that biofertilizer also reduced the damage of insects to plants. Moreover, biofertilizers prepared from aerobic conditions were more effective than anaerobic ones.

Miscellaneous

The use of biofertilizers has also been reported to have positive results on other crops. The field study of Shehata and El-Khawas (2003) on sunflower (*Helianthus annuus*) showed the effectiveness of biofertilizers in natural conditions. Two biofertilizers (Biogien and Microbien) were applied alone and in combination. The application of biofertilizers whether applied in combination or separately significantly enhanced the growth and yield of sunflower. Biofertilizer also enhanced oil and nutrient content of seed as well as nitrogen and nitrogenous compounds and, however, decreased the saturated fatty acids and increased the unsaturated fatty acids.

Nutrition requirement of banana crop is high and use of chemical fertilizers increases the production cost of this crop. Literature showed the beneficial effects of biofertilizer on banana crop. For example, Rivera-Cruz et al. (2008) conducted a study to compare the effectiveness of poultry manure and banana waste as inoculant carrier of bacterial strain and also to establish efficient dose of biofertilizer. They demonstrated that application of biofertilizer not only increased the plant growth but also enhanced the phosphate solubilization, enzyme activities, and aggregate stability. They found that both fertilizers were useful carriers for PGPR. Keeping in view the huge requirement of fertilizers for banana, which is not only costly but has negative impact on environment also, Mia et al. (2010) conducted studies to evaluate the role of PGPR for improving the nutritional requirements of banana. It has been observed that PGPR inoculation with 33 % nitrogen not only enhanced the nutrient supply but also improved root and shoot growth.

It is evident from the above discussion that the use of PGPR as biofertilizers is very useful for promoting plant growth. These biofertilizers are not only useful for fulfilling the nutrient requirement of the plant but also enhance the plant growth by various mechanisms. It has also been observed that although the application of biofertilizers alone can promote plant growth, combined application of biofertilizer with chemical fertilizers can give better results. Therefore, the application of biofertilizer can reduce the use of chemical fertilizer and significant yield could still be obtained.

Phytostimulators

As it was discussed earlier, PGPR use different mechanisms for improving plant growth and development. One of the well-established and common mechanisms used by PGPR is the production of plant growth regulators (PGRs), also called as phytohormones. These PGRs stimulate many plant processes and ultimately enhance growth of plant. Therefore, PGPR owing to their ability to produce phytohormones also act as phytostimulators. The five major plant growth-promoting hormones that play important role in plant growth promotion include auxins, abscisic acid, gibberellins, cytokinins, and ethylene (Arshad and Frankenberger 1998; Garcıa de Salamone et al. 2001; Gutierrez-Manero et al. 2001; Egamberdiyeva 2005).

Although a number of workers have shown that plants have the ability to synthesize these phytohormones endogenously (Moore 1989; McKeon et al. 1995; Walton and Li 1995), PGPR, particularly rhizosphere bacteria, are another potential source of these growth-promoting substances (Frankenberger and Arshad 1995; Patten and Glick 1996). Several workers have reported the production of PGRs from a wide variety of rhizobacteria (Arshad and Frankenberger 1998; Lugtenberg and Kamilova 2009; Ahemad and Kibret 2014). No doubt, chemically synthesized PGRs can also be applied for promoting plant growth; however, microbial-derived PGRs are more effective and economical. Due to low thresholds between inhibitory and stimulatory levels of chemically synthesized PGRs, microbial-produced hormones are more effective by virtue of their continuous and slow release (Khalid et al. 2006).

These phytohormones enhance plant growth not only in normal conditions, but some hormones also play important role by promoting plant growth in unfavorable environment (Barazani and Friedman 1999; Patten and Glick 2002; Raghavendra et al. 2010). For example, indoleacetic acid (IAA) is helpful for alleviating the impact of drought stress on plant growth and development (Leinhos and Bergmann 1995). San-Francisco et al. (2005) while studying the effect of IAA and its precursor on the development, mineral nutrition, and free polyamine content of pepper plants found an increase in spermine and a decrease in putrescine in pepper leaves by the application of IAA. Similarly, abscisic acid (ABA) also considered as an important PGR, playing an important role in the photoperiodic induction of flowering (Wilmowicz et al. 2008), plays central role in stress (Zhang et al. 2006).

In earlier studies, it has been observed that high nitrate concentration is inhibitory to root growth (Zhang et al. 1999; Scheible et al. 1997; Forde 2002); however, the mechanism of this inhibition was not known. Tian et al. (2008) reported that reduced maize growth due to elevated levels of nitrate was due to the low IAA level in the plant root. Salinity is a major constraint to sustainable agriculture, and a decrease in cytokinin supply from root to shoot was also observed under salinity stress (Naqvi and Ansari 1974). Egamberdieva (2009) attributed better growth of inoculated wheat under salinity stress to the production of phytohormones by PGPR. He used PGRs including gibberellins, auxins, zeatin, and ethephon as well as auxin-producing *Pseudomonas* spp. The results showed that PGRs and *Pseudomonas* spp. significantly enhanced the seedling growth under saline conditions compared to control.

Arkhipchenko et al. (2005) observed a positive response on growth of legume fodder by the application of PGPR. They found a significant quantity of L-tryptophan that was the major precursor of IAA (Barazani and Friedman 1999). They demonstrated that this growth promotion was due to IAA. It is also evident from the work of Cassan et al. (2009) who observed that bacterial strains *Azospirillum brasilense* and *Bradyrhizobium japonicum* enhanced the germination and growth of corn and soybean. The effect was positive whether the strains were used singly or in combination. The strains had the ability to produce IAA, gibberellic acid, and zeatin (member of cytokinins). They observed that both strains were able to secrete these hormones into the culture medium, at a concentration that was sufficient to produce morphological and physiological changes in young seed tissues.

Barbieri et al. (1991) and Burdman et al. (1996) reported the effectiveness of *Azospirillum* sp. for enhancing root growth in grasses and legume, respectively, through phytohormone production. Also, IAA-producing *Bradyrhizobium* sp. enhanced total biomass and grain yield in chickpea (Alagawadi and Gaur 1988). The co-inoculated effect of these two strains, *Azospirillum* and *Bradyrhizobium* sp., was evaluated by Molla et al. (2001). In a laboratory study, soybean was co-inoculated with *Azospirillum* spp. (*A. brasilense* and *A. lipoferum*) and *Bradyrhizobium* sp., and its potential for enhancing root growth and nodulation was evaluated. They observed that total root length, root hair, and shoot dry weight significantly increased by co-inoculation. Co-inoculated plants also showed more number of nodules and weight. It was also observed that *A. brasilense* performed better than *A. lipoferum*.

Phytoextraction is an important and attractive approach to clean up the soil environment from toxic heavy metals. The ability of a plant to take up heavy metals depends upon its better root system. The PGRs are helpful in phytoextraction by influencing the root growth and metal availability. IAA is known to induce root growth by enhancing cell division and cell extension (Taiz and Zeiger 2000). It has been observed that chelating agents used to extract metals increase the metal concentration that may become harmful for plant growth (Tandy et al. 2006); however, this negative impact could be eliminated by the application of IAA (Liu et al. 2007). Tassi et al. (2008) found that exogenous PGR based on cytokinins positively enhanced phytoextraction of Pb and Zn and improved biomass production of *Helianthus annuus*.

From the above discussion, it is clear that phytohormones produced by PGPR play an important role for plant growth promotion. These phytohormones accelerate a number of plant processes. A large number of PGPR strains produce different types of growth hormones. These phytohormones are not only helpful for normal growth processes but also protect the plants from negative impact of stress. Owing to the ability of producing hormones, these PGPR strains enhance the plant growth and development under adverse conditions.

Synergistic Application with Symbiotic Bacteria (Co-inoculation)

The effectiveness of PGPR for enhancing plant growth is well documented. These beneficial bacteria can also be used effectively with other microbes for promoting their growth and yield. This co-inoculation is equally effective under normal as well as stress conditions. The study of Mishra et al. (2011) showed the effectiveness of co-inoculation of *Pseudomonas* and *Rhizobium* sp. for growth enhancement and nutrient acquisition of lentil. Although inoculation of bacterial strain singly caused significant effect on growth, nodulation, and nutrient uptake, co-inoculation of *Pseudomonas* with *Rhizobium* recorded maximum increase in nodulation, chlorophyll content, and total iron, nitrogen, and P uptake. This study indicates the synergistic relationship between the strains that is helpful for improving plant growth and also suggested the use of such strains for biofertilizer production. Previous studies have also revealed that combination of microorganisms showed synergistic interaction and was found helpful for enhancing plant growth (Khammas and Kaiser 1992; Belimov et al. 1995; Bashan and Holguin 1997a, b; Bashan 1998). For example, *Azospirillum* is considered as a *Rhizobium* helper, which enhances nodulation and nodule activity and provides resistance in plants against unfavorable conditions (Itzigsohn et al. 1993; Fabbri and DelGallo 1995). Egamberdieva et al. (2010) found that co-inoculation of *Pseudomonas* sp. capable of producing IAA and *Rhizobium* (not producing IAA) enhanced shoot and root dry weight of fodder *Galega* (*Galega orientalis* Lam.) compared to *Rhizobium* alone.

The co-inoculation becomes more essential when a single strain is not so effective in some particular environment. For example, under salinity stress, reduction in nodulation has been observed (Zahran 1999). Nitrogenase enzyme that is a major component of nitrogen fixation is affected by salt concentration (Jofre et al. 1998). Also, under stress environment, elevated level of ethylene is produced that is inhibitory for the overall process of nitrogen fixation (Taiz and Zeiger 2000). This elevated level of ethylene is due to ACC that is the immediate precursor of ethylene. It has been observed that certain PGPR strains contain ACC deaminase enzyme, which could be helpful for reducing the ethylene concentration that causes negative impact on nodulation in legumes under stress environment (Ahmad et al. 2011). It was observed that co-inoculation of *Pseudomonas* and *Rhizobium phaseoli* proved very effective for enhancing growth and nodulation of mung bean under salinity stress. It has been observed that this growth enhancement was equally effective under natural salt-affected field condition (Ahmad et al. 2013a, b). Co-inoculation of *Rhizobium* with two different PGPR strains enhanced the growth of mung bean in saline environment under axenic conditions (Fig. 1.1). This growth enhancement was also observed when same study was conducted in greenhouse (Fig. 1.2). Similarly, Malik and Sindhu (2011) also observed that co-inoculation of *Mesorhizobium* sp. with IAA-producing *Pseudomonas* increased the nodule number and nodule biomass of chickpea (*Cicer arietinum*). The positive results of co-inoculation are not only observed in case of legume but also in nonlegumes. The study of Bano and Fatima (2009) showed that inoculation of *Pseudomonas* and *Rhizobium* proved useful for promoting maize growth under saline conditions by increasing water content and decreasing electrolyte leaking of inoculated plants.

However, for getting significant results, it is necessary to use the partners that have compatibility with each other. In case of noncompatible

Fig. 1.1 Effect of co-inoculation on root/shoot growth of mung bean seedlings under axenic conditions at 6 dS m^{-1} (**Mk20** and **Mk25**: PGPR containing ACC deaminase; **M9** and **M6**: *Rhizobium*)

Fig. 1.2 Effect of co-inoculation on growth of mung bean at 6 dS m^{-1} in pot trial (**Mk25**: PGPR containing ACC deaminase; **M9**: *Rhizobium*)

partner, effective results may not be obtained. Moreover, the kind of plant may also have influence on the performance of inoculation. For example, the study of Rosas et al. (2006) showed that co-inoculation of *Pseudomonas putida* with *Sinorhizobium meliloti* was more effective in case of soybean than alfalfa where *Bradyrhizobium japonicum* was more successful with *P. putida*.

Rhizosphere Bacterial Consortia

Although PGPR inoculation has been proved useful for enhancing plant growth and development in normal and stress conditions, in certain cases particularly in field, inconsistent results are obtained with single inoculum (Lucy et al. 2004; Smyth et al. 2011). This inability of bacteria to show their full potential might be due to certain factors including competition with indigenous population and low-quality inoculum. The use of multi-strain inoculum could be one of the best solutions to this problem. A multi-strain bacterial consortium is a group of different species of bacteria, which act together as a community. The inoculation of *Withania somnifera* (Ashwagandha) with multi-strain consortia of *Azospirillum*, *Azotobacter*, *Pseudomonas*, and *Bacillus* showed significant increase in plant height and root length when compared to uninoculated control (Rajasekar and Elango 2011). For obtaining significant results, it is necessary to use such species which have compatibility with each other in addition to having good growth-promoting traits. Choure and Dubey (2012) suggested some characteristics of a good bacterial consortium. These include bacterial species which are resistant to environmental stress, have natural enzymatic activity and long shelf life, and are nonpathogenic besides having good sustainability and economic feasibility.

The performance of bacterial consortium was more effective when used in a stress environment. The study of Upadhyay et al. (2011) conducted under salinity stress condition showed that dual inoculation enhanced wheat growth by increasing sugar and proline content as well as decreasing sodium content of inoculated wheat. Similarly, Annapurna et al. (2011) demonstrated that single and dual inoculation showed variable response under stress condition. Although single inoculation also proved effective for diluting the negative impact of salinity, the bacterial consortium was more effective for inducing salinity tolerance in wheat. Similarly, the use of more than one bacterial species was also effective for promoting nodulation when bean plants were co-inoculated with *Rhizobium* spp. and *Bacillus polymyxa* (Petersen et al. 1996).

The susceptibility of plant to pathogens is one of the important factors that adversely affect plant growth and development and ultimately cause yield reduction. Although plant pathogens can be controlled effectively by synthetic chemicals, there are certain environmental concerns related to these toxic substances. It has been observed that the use of bacterial consortium is also very effective for promoting plant growth by protecting them from pathogenic diseases. The combined application of *Sinorhizobium fredii* KCC5 and *Pseudomonas fluorescens* LPK2 significantly reduced the wilt disease as reported by Gupta et al. (2002). They demonstrated that the use of microbial consortia could be an effective way for the management of fungal disease. The study of Choure and Dubey (2012) showed that the multi-strain consortium of three bacterial species *P. fluorescens* LPK2, *S. fredii* KCC5, and *A. chroococcum* AZK2 inhibited the conidial and chlamydospore germination of *Fusarium udum* and reduced the *Fusarium* wilt in *Cajanus cajan*. They demonstrated that multi-strain bacterial inoculum can be used effectively for protecting the plant from fungal diseases.

Precursor–Inoculum Interaction

As discussed in the earlier section, PGPR produce a number of phytohormones that stimulate plant growth and development. These growth hormones are also very effective for enhancing plant growth under stress environment. It has been observed that same strains may produce variable amount of phytohormones that could be the reason of inconsistent results obtained under certain conditions. This might be due to certain factors; among them the availability of suitable precursor is one of the primary factors affecting the secretion of these hormones. This drawback can be overcome by the use of specific precursor in addition to inoculation. It has been observed that exogenous application of a precursor increased the magnitude of phytohormone production many fold in culture as well as in soil (Asghar et al. 2000, 2002). This precursor–inoculum interaction is useful for promoting plant growth. A number of studies conducted by various

workers showed that precursor–inoculum interaction significantly enhanced plant growth (Sarwar and Kremer 1995; Zahir et al. 1997; Arshad and Frankenberger 1998). This approach is also effective for controlling the production of a hormone by providing suitable concentration of a particular substrate.

The approach is effective in a number of ways particularly in conditions where the application of a particular hormone is difficult. For example, ethylene is a gaseous hormone that stimulates various physiological processes in plants. The application of ethylene due to its gaseous nature is difficult. However, its precursor L-methionine (L-MET) can release ethylene in the presence of soil microflora therefore stimulating plant growth (Arshad and Frankenberger 1998; Khalid et al. 2006; Mahmood et al. 2008).

L-tryptophan is considered as an efficient precursor for microbial biosynthesis of auxins (Frankenberger and Arshad 1991). Zahir et al. (2005) conducted a study to evaluate the effectiveness of precursor–inoculum interaction for enhancing maize growth. Auxin production by *Azotobacter* was measured in the presence and absence of its precursor L-tryptophan. Results of field trials showed that application of *Azotobacter* alone or in combination with L-tryptophan significantly enhanced maize growth. However, combined application of *Azotobacter* and L-tryptophan caused pronounced effect on grain yield, fresh biomass, and total nitrogen uptake compared to uninoculated control and where sole inoculation was used. This study showed that the application of a precursor along with microbial strain could be more effective. Prior to this, similar results were also been observed in other crops (Zahir et al. 1997, 2000). In an earlier study, Khalid et al. (1999) also reported the growth and yield enhancement of wheat by the application of *Azotobacter* in the presence of L-tryptophan.

Precursor–inoculum interaction is not only effective under normal conditions but is also useful for promoting plant growth under stress conditions. To evaluate the performance of *Rhizobium* for improving growth and yield of mung bean (*Vigna radiata* L.), a pot study was conducted under saline conditions in the presence and absence of L-tryptophan (Zahir et al. 2010). Although salinity reduced the growth of mung bean, application of L-TRP and *Rhizobium* appeared to mitigate the adverse effects of salt stress. It was further observed that combined application of *Rhizobium* and L-tryptophan produced more pronounced effect.

The above discussion shows that although PGPR can enhance plant growth by phytohormone production, in certain cases the expected results are not obtained. This might be due to their inefficient production of these hormones. This low production of phytohormones occurs due to unavailability of a particular substrate. It has been observed that such limitation can be removed by using suitable substrate during inoculation. The use of this technology could be beneficial in the environment where sole bacterial inoculation cannot perform efficiently. The significant results can also be obtained by using multi-strain consortia.

Rhizosphere Bacteria for Improving Stress Tolerance

In arid and semiarid climate, different environmental stresses affect plant growth by affecting a number of physiological processes positively or negatively. These stresses may be abiotic (salinity, drought, temperature, and heavy metal stress) or biotic, i.e., pathogen and disease stress. Stress induces different physiological disorders such as nutrient deficiency, hormonal imbalance, increase in concentration of certain osmolytes, and higher levels of ethylene in plants. High ethylene concentration is one of the contributing factors for premature senescence of different plant parts. Synthesis of ethylene under stress can be suppressed biologically through the action of rhizosphere bacteria that can degrade the ethylene precursor ACC through their enzyme ACC deaminase (Glick et al. 1998). Rhizosphere bacteria have the potential to colonize plant roots under stressed environment. These bacteria lower the endogenous level of ethylene and thus promote plant growth. In addition, these bacteria possess several other traits

like production of different growth regulators such as auxins, gibberellins, cytokinins, and/or polyamines, which directly promote plant growth or indirectly induce systemic resistance. In this section, significant improvement in plant biomass under stress conditions through inoculation with different strains of PGPR has been reviewed and discussed.

Abiotic Stress Regulation

Salinity, drought, temperature, and heavy metals are some of the important abiotic stresses that adversely affect the plant growth one way or the other. Some of the common effects of these stresses on plant growth include hormonal and nutritional imbalances, ion toxicity, and osmotic effect. PGPR have shown to facilitate plant growth indirectly by making the plant more resistant to stress (Glick 2004) or directly by facilitating the uptake of nutrients from the environment, by influencing phytohormone production (e.g., auxin, cytokinin, and gibberellins), by enzymatic lowering of plant ethylene levels, and/or by production of siderophores (Patten and Glick 1996; Kohler et al. 2006; Nadeem et al. 2009; Ahmad et al. 2011). The production of exopolysaccharides and disaccharides by some rhizosphere bacteria may act as protectants against abiotic stresses such as drought, salinity, and temperature stress. For example, some bacteria have the ability to produce trehalose which acts as protectant in plants against abiotic stresses. Trehalose is resistant to acid and high temperature and is a highly stable molecule that can form a gel phase as cells dehydrate, replacing water and, as a result, decreasing damage from drought and salt. Moreover, trehalose also prevents degradation and aggregation of proteins under different stresses. Thus, inoculation of plants with trehalose-producing rhizobacteria confers resistance against stresses (Suarez et al. 2008; Rodriguez-Salazar et al. 2009). The effectiveness of PGPR inoculation to reduce the negative impact of these stresses on plant growth has been reviewed in the following sections.

Salinity Stress

Salinity is one of the most critical constraints hampering agricultural productions in many areas around the world. However, utilization of salt-affected soils for agricultural production is also indispensable to feed the burgeoning population over the globe. Reducing salinity-induced ethylene by any mechanism could decrease the negative impact of salinity on plant growth. Recent studies have revealed that plants inoculated with rhizobacteria were better able to thrive through the salinity stress while demonstrating a normal growth pattern. Hydrolysis of ACC by rhizobacterial strains carrying ACC deaminase activity diluted the adverse effect of salinity by decreasing the ethylene production thus improving seedling growth of plants (Shaharoona et al. 2006; Nadeem et al. 2007, 2009; Ahmad et al. 2011). Reduction in ethylene production due to inoculation with PGPR containing ACC deaminase has been reported in various studies as well (Glick et al. 1998; Indiragandhi et al. 2008).

Inoculation with PGPR containing ACC deaminase has been reported to eliminate or at least reduce the stress-induced ethylene-mediated negative impact on plants (Glick 2005; Safronova et al. 2006). It has been well documented that different bacterial species belonging to different genera such as *Agrobacterium*, *Achromobacter*, *Azospirillum*, *Burkholderia*, *Enterobacter*, *Pseudomonas*, and *Ralstonia*, possessing variable ACC deaminase activity, increased the growth of different crops (Arshad et al. 2008; Nadeem et al. 2010b). Many researchers have reported that exogenous application of ethylene and/or salinity had adverse effect on plant growth; however, inoculation with PGPR containing ACC deaminase improved the plant growth (Shaharoona et al. 2006; Cheng et al. 2007; Nadeem et al. 2009; Ahmad et al. 2011). Improvement in dry matter and salt tolerance of tomato (Mayak et al. 2004a), canola (Cheng et al. 2007), mung bean (Ahmad et al. 2011), wheat (Zahir et al. 2009; Nadeem et al. 2010a), and chickpea (Patel et al. 2012) has been reported due to inoculation with PGPR containing ACC deaminase under stress.

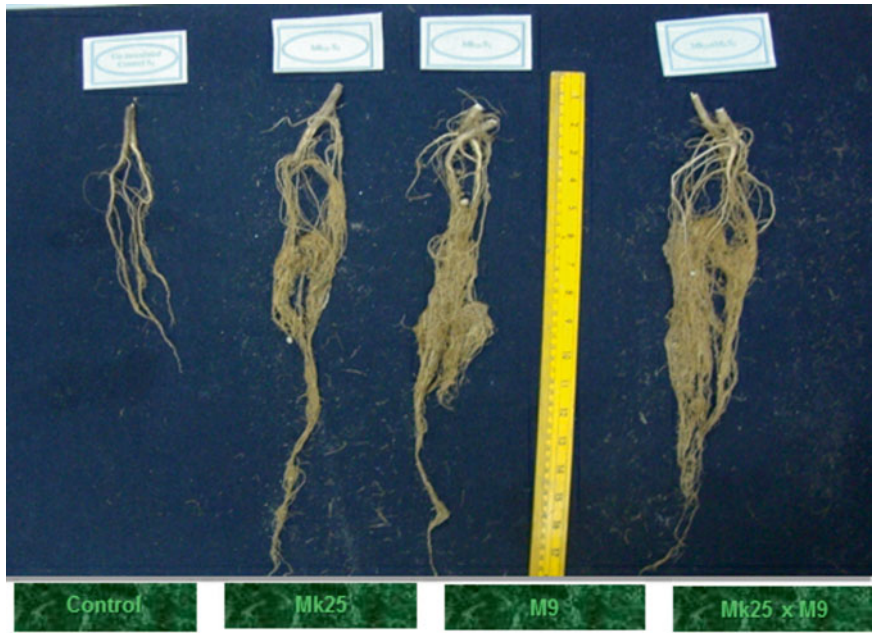

Fig. 1.3 Effect of co-inoculation on root length of mung bean at 6 dS m^{-1} in pot trial (**Mk25**: PGPR containing ACC deaminase; **M9**: *Rhizobium*)

The worth of PGPR strains containing ACC deaminase, i.e., *P. fluorescens*, has been reported by Shaharoona et al. (2008), where they studied that the efficacy of these strains carrying ACC deaminase may likely be due to the reduction of stress-induced ethylene level. It is also evident from a pot study where co-inoculation of *Rhizobium* with a PGPR strain containing ACC deaminase significantly enhanced the root length (Fig. 1.3) might be due to lowering of stress-induced ethylene (Ahmad et al. 2012).

The microbial treatments also reduce the Na^+ uptake by plants and increase the K^+ uptake thus increasing the K^+/Na^+ ratio which otherwise is reduced by salinity (Giri et al. 2007). The rhizobacterial species *Variovorax paradoxus* effectively colonized the plant roots under stressed conditions and hence can be used efficiently for attenuating the growth inhibition of plants under stressed conditions (Belimov et al. 2009a). These PGPR containing ACC deaminase can decrease the stress-induced endogenous ethylene level (Mayak et al. 2004a). The inoculation with *A. brasilense* increases the relative water content in the leaf due to the production of ABA by the bacterial strains thus reducing the ACC level in plants (Cohen et al. 2008).

Mayak et al. (2004a) reported that *Achromobacter piechaudii* having ACC deaminase activity significantly increased the fresh and dry weights of tomato seedlings grown in the presence of NaCl (up to 172 mM). The bacterium reduced the production of ethylene by tomato seedlings, which otherwise was stimulated when seedlings were grown with increasing salt concentrations. However, the sodium content of the plant was not decreased, whereas the uptake of phosphorous and potassium was slightly increased, which might have contributed in part to the activation of processes involved in the alleviation of the adverse effects of salts on plant growth. The bacterium also increased the water use efficiency (WUE) in saline soil and helped in alleviating the salt suppression of photosynthesis. *Pseudomonas fluorescens* strain TDK1 containing ACC deaminase activity enhanced the saline resistance in groundnut plants and increased yield as compared with that inoculated with *Pseudomonas* strains lacking ACC deaminase activity (Saravanakumar and Samiyappan 2007).

Similarly, ACC deaminase bacteria have also been reported to increase the salt tolerance in plants by lowering the synthesis of salt-induced stress ethylene and promote the growth of canola in saline environment (Cheng et al. 2007). Nadeem et al. (2009) observed similar results in case of maize grown under salt stress in response to inoculation with PGPR containing ACC deaminase activity. Hamaoui et al. (2001) reported that inoculation of chickpea with *A. brasilense* significantly reduced the negative effects on plant growth caused by irrigation with saline water (EC, 4.8 dS m^{-1}; 714 mg L^{-1} NaCl) under controlled conditions. They conducted an experiment in a greenhouse and found that the inoculation with *A. brasilense* significantly increased the nodulation and root and shoot development under both normal and saline water conditions. It has been well documented that the PGPR containing ACC deaminase have the ability to tolerate high concentrations of salinity under a wide range of temperature variations (Hontzeas et al. 2005).

Chlorophyll contents in the plant shoot are increased when inoculated with *Pseudomonas* stains having ACC deaminase activity (Glick et al. 1997; Nadeem et al. 2009). This increase in chlorophyll content may be due to the increased photosynthetic leaf area of the plant by inoculation compared to uninoculated control where leaf area was reduced due to salinity stress (Marcelis and van Hooijdonk 1999). Thus, improvement in chlorophyll contents might have resulted in increased photosynthetic rate which ultimately produced more photosynthates, leading to increased yield of crops.

Increased nutrient concentration in maize plants has been reported under salt stress due to bacterial inoculation in crop plants (Hamdia et al. 2004; Nadeem et al. 2009). Inoculation with PGPR containing ACC deaminase significantly increased P and K contents in salt-stressed tomato plants (Mayak et al. 2004a). Similarly, enhanced uptake of N, P, K, and Ca content of soybean due to inoculation with PGPR under salinity stress has been reported by Han and Lee (2005). It has been reported that the root elongation rate and mineral N, P, and K and microelement uptake are consequently improved after *Azospirillum* inoculation (Dobbelaere and Okon 2007). This could result in a generally better mineral nutrition of the plant (such as phosphorous, iron, and molybdenum) essential for rhizobia–nodule formation and nitrogen fixation activities (Burdman et al. 1998). Thus, enhanced nodulation with improved mineral nutrition might have improved the yield of mung bean under salt-affected conditions.

Inoculation with PGPR containing ACC deaminase reduces the absorption and uptake of Na^+. The reduction in Na^+ uptake might be due to the decreased passive flow of Na^+ into the vascular tissues due to more proliferation of roots in inoculated/co-inoculated plants. Similarly, Nadeem et al. (2009) observed that PGPR containing ACC deaminase significantly increased K^+ in maize under salinity stress. Increase in K^+ uptake and decreased Na^+ uptake due to inoculation with PGPR having ACC deaminase have also been reported by Yue et al. (2007). It has been reported that an increase in concentration of K^+ in plants under salt stress could ameliorate the deleterious effects of salinity on growth and yield (Giri et al. 2007). This improvement in ionic/nutritional balance might have resulted into more growth and yield of crop plants.

Different PGPR strains differ in their ability to promote plant growth due to difference in ACC deaminase activity (Shaharoona et al. 2006; Nadeem et al. 2007, 2009). This difference may also be due to the presence of other growth-promoting characters, i.e., chitinase activity, phosphate solubilization, root colonization, etc., in addition to ACC deaminase activity. Mechanisms used by rhizobacteria to induce salinity stress have been summarized in Table 1.1.

Drought Stress

Drought is one of the most important abiotic stresses that have drastic effects on plant growth and productivity by creating dehydration and osmotic stress (Boyer 1982; Glombitza et al. 2004). There are a number of chemicals (Mascher et al. 2005; Jaleel et al. 2007; Liu et al. 2009), PGRs (Lu et al. 2009; Yuan et al. 2010), and alternative products which can be used to reduce the deleterious effects of drought on plant growth

Table 1.1 Abiotic stress tolerance in plants induced through rhizosphere bacteria

Crop	Rhizobacterial strain	Mechanism used/processes regularized	Reference
Salt stress			
Chickpea	*P. putida* MSC1 and *Pseudomonas pseudoalcaligenes* MSC4	Phosphate solubilization, siderophore production, and IAA production	Patel et al. (2012)
Pepper	*Bacillus aryabhattai* RS341 and *Brevibacterium epidermidis* RS15	Suppression of ethylene production due to ACC deaminase activity	Siddikee et al. (2012)
Eggplant	*Xanthobacter autotrophicus* BM13, *Enterobacter aerogenes* BM10, and *Bacillus brevis* FK2	Increase in potassium, K^+–Na^+ ratio, and K^+–Na^+ selectivity in the eggplant shoots	Abd-El-Azeem et al. (2012)
Maize	*P. fluorescens* S20, and *Enterobacter aerogenes* S14	ACC deaminase activity, siderophore production, enhancing microbial growth in the rhizosphere	Ahmad et al. (2014)
	A. chroococcum	Phosphate solubilization, auxin production, and catalase activity	Rojas-Tapias et al. (2012)
	E. aerogenes and *P. fluorescens*	ACC deaminase activity	Zahir et al. (2012)
Cotton	*P. putida* Rs-198	Increased absorption of Mg^{2+}, K^+, and Ca^{2+} and decreased uptake of Na^+ from the soil and also improved production of endogenous indoleacetic acid (IAA) content and reduced abscisic acid (ABA) content of cotton	Yao et al. (2010)
Arabidopsis thaliana	*B. subtilis* GB03	Soil bacteria-induced salt tolerance in plants by tissue-specific regulation of the sodium transporter HKT1	Zhang et al. (2008)
Cucumber and tomato	*Pseudomonas chlororaphis* TSAU13	Protecting plants against soilborne pathogens and regulation of phytohormone production	Egamberdieva (2012)
Sunflower	*P. fluorescens* biotype F and *P. fluorescens* CECT 378^T	Indoleacetic acid and siderophore production and improvement in K^+–Na^+ ratio	Shilev et al. (2012)
Mung bean	*Pseudomonas syringae* Mk1, *P. fluorescens* Mk20, and *P. fluorescens* Biotype G, Mk25	Inhibition of ethylene due to ACC deaminase activity, catalase activity, chitinase activity, phosphate solubilization, root colonization, siderophore production	Ahmad et al. (2012, 2013a, b)
Wheat	*P. putida* (W2) and *P. fluorescens* (W17)	Inhibition of ethylene due to ACC deaminase activity, phosphate solubilization, root colonization, siderophore production	Nadeem et al. (2010a)
Drought stress			
Wheat	*Burkholderia phytofirmans* PsJN	Modulation of metabolism, improving ionic balance	Naveed et al. (2014a)
	Bacillus amyloliquefaciens 5113 and *A. brasilense* NO40	Improvement in homeostatic mechanisms and may be due to a combination of morphological, physiological, and metabolic effects	Kasim et al. (2012)
	Azospirillum	Morphological modifications of the coleoptile xylem architecture, upregulation of its own indole-3-pyruvate decarboxylase gene, and enhanced bacterial IAA synthesis	Pereyra et al. (2012)

(continued)

Table 1.1 (continued)

Crop	Rhizobacterial strain	Mechanism used/processes regularized	Reference
Rice	*A. brasilense*	AM colonization through promotion of fungal propagule germination, stimulation of mycelial growth, or changes in the root architecture through the production of growth factors	Ruiz-Sanchez et al. (2011)
Maize	*B. phytofirmans* PsJN, *Enterobacter* sp. FD17	Modulation of metabolism through endophytic colonization	Naveed et al. (2014b)
	P. putida	Suppression of ethylene production due to ACC deaminase activity	Vilchez and Manzanera (2011)
	Pseudomonas entomophila BV-P13, *Pseudomonas stutzeri* GRFHAP-P14, *P. putida* GAP-P45, *P. syringae* GRFHYTP52, and *Pseudomonas monteilii* WAPP53	Regulation of activities of antioxidant enzymes, ascorbate peroxidase (APX), catalase (CAT), glutathione peroxidase (GPX)	Sandhya et al. (2010)
Chickpea	*Paenibacillus lentimorbus* B-30488	Enhanced growth due to biofilm formation	Khan et al. (2011)
Cucumber	*Bacillus cereus* AR156, *B. subtilis* SM21, and *Serratia* sp. XY21	Reductions in the leaf monodehydroascorbate (MDA) content and extent of the peroxidation of plasma lemma, protection of roots from detrimental effects of drought stress, and increase in activity of antioxidant enzymes and chlorophyll contents	Wang et al. (2012)
Ornamental plants	*V. paradoxus* 5C-2	Reduction in endogenous production of stressed ethylene due to ACC deaminase activity	Sharp et al. (2011)
Green gram (*V. radiata*)	*P. fluorescens* Pf1	Induced systemic tolerance by regulating the activity of catalase and peroxidase	Saravanakumar et al. (2011)
Sugarcane	*Azospirillum* sp.	Production of IAA and enhanced microbial activity in the rhizosphere of inoculated plants	Moutia et al. (2010)
Heavy metal stress			
Brassica juncea	*Enterobacter* sp. CBSB1	The introduced glutathione synthase gene *gcsgs* into endophytic strain CBSB1 improved phytoremediation efficiency of host plant	Qiu et al. (2014)
Spartina densiflora	*Aeromonas aquariorum* SDT13, *Pseudomonas composti* SDT3, and *Bacillus* sp. SDT14	Consortium was able to twofold increase seed germination and to protect seeds against fungal contamination in polluted estuaries	Andrades-Moreno et al. (2014)
Agrostis capillaris	*Pantoea* sp. and *Bacillus* sp.	Inoculation with seed endophytes significantly improved plant growth and Cd uptake	Truyens et al. (2014)
Wheat	*P. putida* KT2440	Expression of phytochelatin synthase gene in plants resulted in high heavy metal resistance and high accumulation capacity of Cd	Yong et al. (2013)

(continued)

Table 1.1 (continued)

Crop	Rhizobacterial strain	Mechanism used/processes regularized	Reference
Mirabilis jalapa	*P. fluorescens* strains AA27, MO49, CD1, and CC45	Significantly promoted leaf uptake for Cr, Cu, and Zn	Petriccione et al. (2013)
Pearl millet	*Pseudomonas*, *Citrobacter*, *Acinetobacter*, *Serratia*, and *Enterobacter*	Phosphorus solubilization, IAA production, tolerance to Zn toxicity, temperature stress, and salinity stress	Misra et al. (2012)
Chinese brake (*Pteris vittata* L.)	*Rhodococcus* sp. TS1, *Delftia* sp. TS33, *Comamonas* sp. TS37, *Delftia* sp. TS41, and *Streptomyces lividans* sp. PSQ22	Enhanced population of rhizosphere microbes and increase in arsenic bioavailability through reduction of arsenate	Yang et al. (2012)
Canola and barley	*P. putida* 11, *P. putida* 4, and *P. fluorescens* 169	IAA production, siderophore production, and ACC deaminase activity	Yancheshmeh et al. (2011)
Temperature stress			
Vitis vinifera L.	*B. phytofirmans* strain PsJN	The endophytic colonization of PsJN improved plant photosynthesis and regulated carbohydrate metabolism compared to control	Fernandez et al. (2012)
	B. phytofirmans PsJN	Stress-related gene expression, modulating carbohydrate metabolism and metabolite accumulation	Theocharis et al. (2012)
Wheat	*Pseudomonas lurida* M2RH3	Production of siderophores, IAA, and HCN and solubilization of phosphorus	Selvakumar et al. (2011)
	Pseudomonas sp.	Bacterization improved the root/shoot length and biomass and the level of cellular metabolites compared to control	Mishra et al. (2011)

and physiology. However, work has been carried out, and a number of rhizobacterial species have been explored which have the ability to elucidate the deleterious effects of drought on plant growth due to ACC deaminase activity (Zahir et al. 2008; Belimov et al. 2009a, b) and production of PGRs along with some other mechanisms (Benabdellah et al. 2011). Literature which supports the various mechanisms used by rhizosphere bacteria to induce drought stress tolerance in plants has been reported in Table 1.1.

Rhizosphere bacteria induce abiotic stress tolerance in plants by imposing physical and chemical changes which is called as "induced systemic tolerance" (IST) (Yang et al. 2008) through various mechanisms. Induced drought tolerance in *Arabidopsis thaliana* has been reported due to stimulation of transcription of a drought-response gene, Early Responsive to Dehydration 15 (ERD15), and of an ABA-responsive gene, RAB18, through inoculation with rhizobacterial strain *Paenibacillus polymyxa* (Timmusk and Wagner 1999). Induction of systemic tolerance to drought in many plant species by the action of ACC deaminase-containing rhizobacteria has also been a well-established fact. For example, a significant increase in growth of tomato and pepper seedlings has been reported through inoculation with ACC deaminase-containing PGPR strain *Achromobacter piechaudii* ARV8 which otherwise was decreased due to drought stress. Moreover, ethylene production in tomato seedlings under drought was also decreased due to inoculation (Mayak et al. 2004b). An increase in N, P, and K concentrations in lettuce plants inoculated with *Bacillus* sp. under drought stress has been reported by Vivas et al. (2003).

Fig. 1.4 Impact of inoculation on cucumber growth under drought stress (**Level 2**: water stress by applying water of 70 % field capacity)

Rhizosphere bacteria also induce drought tolerance in plants when applied in combination with mycorrhizal and rhizobial strains. For example, co-inoculation of two rhizobacterial strains *P. polymyxa* (DSM 36) and *P. polymyxa* Loutit (L) with *Rhizobium tropici* (CIAT 899) has pronounced effects in alleviating the drought stress in the common bean (*Phaseolus vulgaris* L.) as compared to sole inoculation (Marcia et al. 2008). Similarly, co-inoculation of rhizobacterial strain *Pseudomonas mendocina* with two arbuscular mycorrhizal strains *G. intraradices* and *G. mosseae* was more effective to increase phosphatase activity, osmolytes regulation, and the activity of other stress enzymes in lettuce leaves grown under drought stress (Kohler et al. 2008). Similarly, some bacteria have the ability to induce drought stress in crop plants due to production of EPS. For example, Grover et al. (2011) reviewed the use of various strains of *Pantoea*, *Rhizobium*, *Pseudomonas*, *Achromobacter*, *Azospirillum*, and *Bacillus* in wheat and sunflower which showed their ability to induce drought stress due to EPS production ability. Rhizobacteria could play a significant role in stress management. Thus, these bacteria can successfully be used to improve drought stress tolerance in crop plants. In a greenhouse study conducted under water stress conditions, inoculation with PGPR enhanced the growth of cucumber compared to uninoculated control (Fig. 1.4).

Temperature Stress

Plants are also sensitive to fluctuation in temperature and heat stress (Mendelsohn et al. 1994; Robertson et al. 1998). Like other environmental stresses, temperature stress also affects plant growth, which might be due to the production of elevated level of ethylene (Cheikh and Jones 1994). Reports are available in literature (Table 1.1) showing that ACC deaminase also protects the plant from temperature stress (Bensalim et al. 1998; Ait Barka et al. 2006). Ethylene and its precursor ACC also have a potential role in flower senescence, leaf abscission, and wilting of flowering species (Woltering and van Doorn 1988; Reid and Wu 1992). Ethylene production decreases the shelf life of flowers that is a major impediment in the success of flowering business (Saleem et al. 2007). Rhizobacteria containing ACC deaminase can also enhance the shelf life of flowers by ameliorating temperature stress (Nayani et al. 1998).

Heavy Metal Stress

Restoration and remediation of metal-polluted soils through phytoremediation is a safe and environment-friendly approach which has gained the attention of scientists (Wu et al. 2006). Reports are available on the use of hyperaccumulating plants for the remediation of heavy metal-polluted soils (Belimov et al. 2005; Zaidi et al. 2006). However, with an increase in metal concentration in plants, the growth decreases due to impaired metabolic activity. Heavy metals enhance the ethylene synthesis that ultimately affect the plant growth in a number of ways (Safronova et al. 2006; Arshad et al. 2008). In addition to an increase in ethylene concentration, heavy metals also cause iron deficiency and evolution of active oxygen species (Burd et al. 1998, 2000; Buchanan 2000). Heavy metal stress tolerance in plants can be induced by a number of abiotic and biotic sources. Biotic elicitors, however, are more beneficial than abiotic as these approaches are safe and environmental friendly. Therefore, the use of rhizosphere bacteria to induce the heavy metal tolerance in plants has gained the attention of scientists as an emerging field of research. Plant–microbe interactions in the rhizosphere have the potential to improve biomass production and heavy metal stress tolerance of crop plants (Glick 2003).

Rhizobacteria may improve plant growth through siderophore production, synthesis of phytohormones and enzymes, as well as phosphate solubilization (Verma et al. 2001). The modulation of high ethylene concentration in plants produced under stressed conditions by some rhizobacteria having ACC deaminase activity may also mediate in heavy metal stress tolerance in crop plants. It is well documented that rhizobacteria containing ACC deaminase activity can improve plant growth and heavy metal stress tolerance in plants in metal-contaminated soils (Belimov et al. 2005; Madhaiyan et al. 2007; Kumar et al. 2008; Belimov and Wenzel 2009).

The rhizosphere bacteria also promote nodulation in contaminated soils. The bacterial strain isolated from Cd-contaminated soil increased the nodulation in clover plants (Vivas et al. 2005) due to accumulation of heavy metal by PGPR in their cells and, thus, reduced the bioavailable Cd concentrations, thereby reducing its uptake by plants and rhizobia. This eliminated Cd toxicity and increased nodulation. The other aspects of this increased nodulation may be because of the enhanced enzymatic activity in the soil (phosphatase, dehydrogenase, etc.) and auxin production by PGPR strains.

Biotic Stress Regulation

Rhizosphere bacteria indirectly influence the plant growth by inhibiting the growth of certain pathogenic organisms by producing antagonistic substances, inducing resistance against pathogens, or releasing compounds against some pests (Glick 1995). The indirect role of rhizobacteria for growth promotion and as defensive agents against diseases and insect pests has been of great importance in modern agriculture in addition to their function as direct growth promoters which has already been well documented (Table 1.2).

Disease Control

Bacteria that are used to control plant diseases and insect pests are called as biocontrol agents, while those having antagonistic properties against certain pathogenic microbes are called as antagonists (Beattie 2006; Glick 2012). As antagonists to plant pathogens, these bacteria can synthesize certain hydrolytic enzymes such as proteases, glucanases, chitinases, cellulases, and lipases. These enzymes hydrolyze the fungal cells (Singh et al. 1999; Frankowski et al. 2001; Kim et al. 2008; Neeraja et al. 2010; Maksimov et al. 2011). Also, they colonize the root surface and rhizosphere where they compete with pathogenic organisms for nutrients and space (Stephens et al. 1993; Kamilova et al. 2005; Compant et al. 2005; Lugtenberg and Kamilova 2009). Another important mechanism is that they cleave the ACC, the immediate precursor of ethylene, into ammonia and α-ketobutyrate through their ACC deaminase activity, thus regulating the production of ethylene (Glick et al. 1998; Mayak et al. 2004a). Furthermore, these bacteria also produce siderophores and antibiotics (Glick 2012). So, rhizosphere bacteria can successfully be used for

Table 1.2 Biotic stress tolerance in plants induced through rhizosphere bacteria

Crop	Stress	Rhizobacterial strain	Mechanism used/processes regularized	Reference
Tobacco	Blue mold	*S. marcescens* 90–116, *Bacillus pumilus* SE 34	Induced systemic resistance due to increased level of salicylic acid and higher activity of β-1,3-glucanase	Zhang et al. (2002)
	Tobacco necrosis virus	*P. fluorescens*	Salicylic acid-dependent activation of PR-1 gene	Park and Kloepper (2000)
Groundnut	*Rhizoctonia bataticola*	*Pseudomonas* sp.	Production of hydroxamate-type siderophores, hydrocyanic acid, and IAA	Gupta et al. (2002)
Chickpea	*Fusarium avenaceum*	*Enterobacter* sp.	ACC deaminase activity and production of IAA and siderophores	Hynes et al. (2008)
Tea	Brown root rot disease	*Ochrobactrum anthropi* TRS-2	Production of siderophores, chitinase, β-1,3-glucanase, peroxidase and phenylalanine ammonia lyase activity	Chakraborty et al. (2009)
Pepper	*Myzus persicae*	*B. subtilis* G803	Suppression of growth due to production of antibiotics	Kokalis-Burelle et al. (2002)
	M. persicae	*B. cereus* MJ-1	Production of gibberellins	Joo et al. (2005)
Tomato	*Phytophthora infestans*	*Bacillus* spp. and *Paenibacillus* sp.	Inhibition of fungal growth due to production of antifungal metabolites and catalase activity	Lamsal et al. (2013)
	Tomato wilt disease caused by *Fusarium oxysporum* f. sp. *lycopersici*	*B. subtilis* S2BC-1 *B. subtilis* GIBC-Jamog	Enhanced activities of chitinases and β-1,3-glucanase and higher intensity of peroxidase isoforms	Shanmugam and Kanoujia (2011)
	Botrytis cinerea	*Bacillus subitus* S499	Activation of lipoxygenase induced by surfactin-overproducing bacteria	Ongena et al. (2007)
Wheat	*Microconidia nivale*	*P. fluorescens*	Production of siderophores and antibiotics	Amein et al. (2008)
	F. oxysporum, *Fusarium culmorum*, *Fusarium solani*, *Pythium ultimum*, *Alternaria alternata*, *B. cinerea*, *Phytophthora cryptogea*	*Acinetobacter*, *Pseudomonas*, *Staphylococcus*, *Bacillus*, *Enterobacter*, *Pantoea*, *Alcaligenes*	Antagonism	Egamberdieva et al. (2008)
	Take-all disease caused by *Gaeumannomyces graminis*	*B. pumilus* 7 km	Stimulation of plant's defensive mechanisms due to increased activities of soluble peroxidase (SPOX), ionically cell wash peroxidase (CWPOX), β-1,3-glucanase, β-1,4-glucanase and enhanced phenolic contents	Sari et al. (2007)

(continued)

Table 1.2 (continued)

Crop	Stress	Rhizobacterial strain	Mechanism used/processes regularized	Reference
Canola	*Sclerotinia sclerotiorum*	*P. chlororaphis*	Enhanced activities of chitinases and β-1,3-glucanase	Fernando et al. (2007)
	Blackleg disease caused by *Leptosphaeria maculans*	*B. cereus* DFE4	Production of lipopeptide antibiotics iturin A, bacillomycin D, and surfactin	Ramarathnam et al. (2011)
		B. amyloliquefaciens DFE16		
Potato	*Fusarium sambucinum*, dry rot	*Serratia plymuthica 5–6*	Production of antifungal metabolites, competition for nutrients and space, and induction of resistance mechanisms in the host	Gould et al. (2008)
Cotton	Cotton root rot caused by *F. oxysporum*	*M. phlei* MbP18	Production of phytohormones, antibiosis, and competition for nutrients and niche	Adesemoye and Egamberdieva (2013)
		B. polymyxa BcP26		
		B. amyloliquefaciens BcA12		
		P. alcaligenes PsA15		
Cucumber	Cotton aphids	*Bacillus* sp.	Production of phytohormone and alteration of metabolic pathways	Stout et al. (2002)
Common bean	*B. cinerea*, *Colletotrichum lindemuthianum*	*P. putida* WCS358	Siderophore-mediated competition for iron and induced systemic resistance	Meziane et al. (2005)
Potato, canola	*P. infestans* US-8	*Bacillus*, *Pseudomonas*, *Rahnella*, and *Serratia*	Production of antibiotics and induction of plant defense systems	Daayf et al. (2003)

the biocontrol of soilborne plant pathogens (Sturz et al. 2000; Compant et al. 2005). These bacteria can also be used in combination with mycorrhizal fungi. For example, the growth of root pathogens like *Fusarium* and *Cylindrocarpon* spp. was inhibited when *Bacillus subtilis* was inoculated in combination with *Laccaria proxima* and *Suillus granulatus* (Schelkle and Peterson 1996).

Indirectly, rhizosphere bacteria have the ability to prevent the proliferation of plant pathogens through the synthesis of different antibiotics (Raaijmakers et al. 2002; Compant et al. 2005; Mazurier et al. 2009). Some strains also have the ability to synthesize hydrogen cyanide in addition to the production of antibiotics which can react synergistically with antibiotics in controlling plant pathogens. Some of the mechanisms that are used by PGPR as biocontrol of plant pathogens have been reviewed in Table 1.2.

Pest Resistance

Rhizosphere bacteria also have the ability to induce systemic resistance in plants. The growth and development of insects is affected by *P. fluorescens* at all growth stages. The growth of larvae of corn earthworm, *Helicoverpa zea*, was affected by *Pseudomonas maltophila* that causes more than 60 % reduction in adult emergence; also, the emerged pupae and adults from infected larvae were smaller and weak (Bong and Sikorowski 1991). In another study, conducted on cotton plants treated with *Pseudomonas gladioli*, the growth and development of *Helicoverpa armigera* was greatly affected which may be due to an increase in their polyphenol and terpenoid content induced by rhizobacteria (Qingwen et al. 1998). Zehnder et al. (1997) while studying the effect of rhizobacterial strains as inducers of systemic resistance in cucumber reported that *P. putida*

strain 89B-27, *Serratia marcescens* strain 90–166, *Flavimonas oryzihabitans* strain INR-5, and *Bacillus pumilus* strain INR-7 significantly reduced the population of *Acalymma vittatum* and *Diabrotica undecimpunctata howardi* on cucumber. But these strains varied in their efficacy to control the insect pests which might be due to difference in effectiveness of these strains as rhizosphere colonizers. Genetically engineered strains with induced insecticidal crystal protein have more potential as biocontrol agents of Lepidoptera insect pests. Transgenic *Pseudomonas cepacia* strain 526, when genetically modified with the crystal protein gene, showed insecticidal activity against tobacco hornworm (Stock et al. 1990). Thus, rhizobacteria can be used as effective biotechnology to control insect pests, and this technology has a prosperous future in sustainable agriculture. Certain bacteria can also be used for inhibiting weed growth owing to the production of certain compounds like hydrogen cyanide (Kremer 2007).

The work reviewed in the above sections indicates that PGPR are using different mechanisms to enhance plant growth and development. Some of these mechanisms are general, while others are specific. By these and other unknown mechanisms, PGPR promote plant biomass and yield under stress conditions by creating a favorable environment that is conducible for plant growth in adverse conditions. Due to root colonization ability and their interaction with plant and other microbial populations, PGPR have great potential to improve root growth, enhance biomass yield, and increase crop yields substantially.

Genetically Engineered Rhizobacteria for Biomass Production and Stress Tolerance Improvement

A number of studies reviewed in the above sections showed the tremendous performance of PGPR for improving plant growth and development under normal as well as stress conditions. This growth enhancement is due to their number of well-defined growth-promoting traits. The occurrence of these growth-promoting traits in bacterial strains is common; however, the presence of all these traits in a single species may be a rare one. Although a number of bacterial strains are considered as PGPR owing to the presence of one or more traits, these bacterial strains may also lack some particular traits that could be beneficial for promoting plant growth under a particular environment (Nadeem et al. 2012). Through biotechnological and molecular approaches, genetically engineered bacteria can be produced expressing some particular traits.

The significant results obtained through microbial inoculation under laboratory and natural field conditions motivate the scientists to develop genetically engineered bacteria expressing a gene of the particular trait. The performances of these bacteria have been evaluated for promoting growth and yield. It has been observed that not only the wild strain but also the genetically engineered strain significantly enhanced the plant growth (Shah et al. 1998; Nascimento et al. 2012). For example, the rhizobacteria containing ACC deaminase are very helpful for promoting plant growth under stress conditions by reducing the negative impact of stress-induced ethylene (Glick et al. 2007; Nadeem et al. 2010a). However, certain bacterial strains do not have such activity and are not able to promote plant growth due to the inability to reduce the elevated level of ethylene that is produced under stress conditions. Efforts have been made to introduce ACC deaminase gene in such bacteria and then observe their ability for promoting plant growth. The work of Shah et al. (1998) showed that cloned ACC deaminase gene can be expressed in *Escherichia coli* enabling this bacterium to grow on ACC as a sole source of nitrogen and having ability to promote root elongation of canola seedlings. The presence of heavy metals and other toxic elements affects the plant growth. Even in certain cases it has been observed that in the presence of toxic compounds, PGPR are unable to promote plant growth (Davison 2005; Pimentel et al. 2011). The growth-promoting ability of PGPR in contaminated environment can be enhanced if inoculated strains have both growth-promoting traits and contaminant-degrading ability. This can be achieved by constructing genetically engineered bacteria having contaminant-degrading

ability and well-established growth-promoting traits. Keeping in view of these, Yang et al. (2011) studied the growth-promoting ability of genetically modified bacteria for enhancing maize growth in the presence of phenol. They used two strains for the construction of a recombinant. One was phenol-degrading *Pseudomonas aeruginosa* SZH16 but did not have growth-promoting ability, and the other was *P. fluorescens* having growth-promoting ability but did not have phenol-degrading ability. The modified strain not only enhanced maize growth but also degraded phenol. Similar results were also observed by Taghavi et al. (2005) where genetically modified endophyte *B. cepacia* BU0072 enhanced the root and leaf mass of yellow lupin in the presence of toluene. Also, endophytic *P. putida* VM1441 protected the pea plant from toxic effect of naphthalene as well as enhanced the efficiency of naphthalene removal (Germaine et al. 2009).

The genetically modified bacteria have also proved useful for protecting the plants from diseases. The biocontrol ability of a PGPR strain can be enhanced by insertion of a functional gene. For example, genetically modified *P. fluorescens* SBW25 was more efficient in reducing damping-off disease in pea than the wild-type strain (Timms-Wilson et al. 2000). This strain was not previously known to produce antimicrobial substances. Similar results were also observed by Bainton et al. (2004) where genetically modified *P. fluorescens* SBW25 enhanced the disease resistance ability of pea plant, although the wild type also had biocontrol ability but reduced number of emerged pea seedlings that did not occur in the case of modified strain.

Concluding Remarks and Future Prospects

The above-discussed review indicates that PGPR can be used effectively for promoting plant growth and development. It has been observed that due to effective root colonization and their interaction with plant and other microbial populations, PGPR have great potential for improving root growth, enhancing biomass yield, and increasing crop yields. The PGPR are equally effective for enhancing growth under control as well as natural conditions. In addition to normal growth-promoting traits, the PGPR also protect the plant from certain biotic and abiotic stresses by using some particular mechanisms, for example, the presence of ACC deaminase enzyme and the production of EPS are one of the very important mechanisms that play a key role for reducing the negative impact of salinity and drought. Through these and other unknown mechanisms, PGPR not only promote plant biomass yield in normal conditions but also create a favorable environment that is conducible for microbes themselves as well as for plant growth in adverse conditions.

One of the drawbacks of this technology which is reported by some of the workers is the lack of reproducible results. It is evident from the above discussion that such limitation can be minimized through precursor–inoculum interaction and by multi-strain bacterial consortium. Significant growth enhancement can be obtained through precursor–inoculum interaction in an environment where a particular substrate is missing or present in low quantity to show full potential of bacterial strains. Similarly, where a single strain was not so effective for providing desired results particularly under stress environment, the use of multi-strain inocula could be one of the better strategies to promote plant growth.

Although the above-discussed review clearly indicates the effectiveness of PGPR for enhancing plant growth and development under stress conditions, certain areas need further research in this regard. The selection of a suitable strain keeping in view the particular problem is very important. For example, the negative impact of stress-induced ethylene can be minimized by the inoculation of PGPR containing ACC deaminase activity. Similarly, a strain containing ACC deaminase activity and EPS-producing ability can be used effectively for protecting the plant from stress-induced impact of salinity and drought. However, a biocontrol strain can be considered as the best if it has fungal-degrading enzymes or other related mechanisms like inducing systemic resistance.

The use of multi-strain inocula is a good strategy where a single strain is not able to give desired results; however, the compatibility of strains with each other is a very important aspect to obtain better results. The selection of suitable and compatible strains is one of the prerequisites for the success of this technology. The significant results can only be obtained by selecting PGPR strains having multiple traits as well as using these strains in proper combination. It is also important to examine the availability of suitable substrates for microbial growth. Significant results can only be obtained if suitable substrates are available for bacteria; otherwise, precursor–inoculum interaction could be a better strategy under such conditions.

The literature shows that genetically engineered bacteria can also be used effectively for promoting plant growth under normal and stress conditions; however, this aspect needs some comprehensive research. The effectiveness of this population under natural field conditions is not much evaluated. Further, the behavior of such genetically modified bacteria in soil environment and their impact on microbial community structure need comprehensive research.

In spite of a number of studies that show the effectiveness of PGPR for enhancing plant growth and development, the field application of these bacteria is limited. This might be due to nonavailability of a suitable carrier material and/or method of application. This aspect needs much research so that their application can be made easily as well as effectively. At present, one of the better ways of PGPR application is their use through biofertilizers. Although certain studies have shown the effectiveness of these biofertilizers for enhancing crop production, this aspect still needs much research. There are certain queries about the use of biofertilizers that are related to their shelf life and effectiveness in the natural field conditions. Moreover, the use of more than one strain which have compatibility with each other is another important aspect that should be kept in mind during biofertilizer preparation. This aspect also needs research so that suitable strains can be used effectively to maximize the benefits that can be obtained on sustainable basis.

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Biopesticides: Where We Stand?

2

Jitendra Mishra, Sakshi Tewari, Sachin Singh, and Naveen Kumar Arora

Abstract

Chemical pesticides are well known for their effective role in disease management because not only they act on a broad host range but production technology is also less expensive. However, the devastating part is their huge negative impact on the environment including the living beings of the planet. In spite of this, in the absence of suitable alternative, the use of synthetic pesticides has dominated around the globe. By the advent of greener approach of developing and using biopesticides, the situation is gradually changing but in fact can move far more swiftly in this direction which will be sustainable and eco-friendly. Although biopesticides are slowly replacing the chemical pesticides, a complete global look at the scenario indicates that the former and particularly the industries based on them are still in an insecure position in comparison to the chemicals which rule the agriculture. We can say that the biopesticides, although show a great promise, have not come up to the desired level so as to displace the dominance of chemicals. In this chapter, the global scenario of biopesticides is discussed emphasizing upon the current demand, use, constraints, and remedies.

Introduction

Two-thirds of today's world population depends upon agriculture for livelihood, but nowadays, growth and production of agricultural crops are getting hampered day by day (Elumalai and Rengasamy 2012). When farmers see their agricultural crops declining in yield and production, they often expect a dramatic, magical treatment to make them lush, green, and healthy again, so that the productivity increases. As a result, they start using chemical pesticides, disregarding their future effects. The extensive use of these synthetic organic chemicals in the past decades has led to a number of long-term environmental problems (Arora et al. 2012). Keeping all these

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facts in mind, a very big challenge in the new millennium is to produce more and more food from shrinking per capita arable land, keeping the environment safe and innocuous.

As agricultural production intensified over the past few decades, producers became more and more dependent on agrochemicals. The conventional chemical pesticides have although enhanced the food production, but have also adversely affected the environment and nontarget organisms. Chemical fertilizers and pesticides are continuously accumulating in the environment, harming the ecosystem, causing pollution, and inflicting diseases at alarming levels (Gerhardson 2002; Arora et al. 2010). The heavy use of pesticides has already caused grave damage to health, ecosystems, and groundwater. Many of the pesticides currently being used have the tendency to survive in plants for a long time. They also enter the food chain and are found in meat and dairy products and remain as residue in the soil and ecosystem for long durations.

Therefore, it is very urgent to identify alternatives to chemical pesticides for plant protection without sacrificing the productivity and profitability of agriculture. Due to the side effects of chemical pesticides, sustainable crop production through eco-friendly management is essentially required in the present scenario.

Biopesticides offer powerful tools to create a new generation of sustainable agriculture products. They are the most likely alternatives to some of the most problematic chemical pesticides currently in use. Biopesticides offer solutions to concerns such as pest resistance, traditional chemical pesticides, and public concern about side effects of pesticides on the surrounding environment and ultimately on human health. The overriding challenge for the biopesticide industry is to live up to the promises and expectations of the end users or the market and public as whole. There are unanswered questions and unexamined assumptions about these biological and eco-friendly alternatives. Challenges to biopesticides stem from questions about their efficacy and safety, public and grower confusion about the spectrum of biopesticide products in the market, and current market conditions that paradoxically both hinder and favor the field's growth.

The aim of the review is to highlight the present global scenario of biopesticides, including its market, availability, and usefulness. Besides this, the compilation also addresses the possible constraints and dilemmas associated with biopesticides throughout the globe and the direction to what should be explored in the future to uplift the stature. Biopesticides offer an environmentally sustainable approach to increase crop production and health, contributing substantially in making the twenty-first century the age of biotechnology hence every effort should be made to enhance their use and popularity.

Shift of Wheel from Pesticides to Biopesticides

It is well known that there have been some discoveries in the past which not only have changed the life of man but also had major influence on the globe, and a very well-known chemical pesticide para-dichlorodiphenyltrichloroethane (DDT) was one of them (West and Campbell 1946). After World War II, its discovery was considered as important as that of penicillin (Felix 1958). In one way when penicillin was saving the life of millions from life-threatening bacterial infections, DDT was protecting crops from a variety of pests. Although for a few years the glory of DDT was extolled by everyone, but soon the whole picture started to change. For the first time, Rachel Carson, a marine biologist and conservationist, stated in her book *Silent Spring* that we are paying much more than what we are getting to get rid of mosquitoes (Carson 1962). Finally in the USA, DDT was banned in 1972 (Griswold 2012). There are myriads of incidences dealing with DDT poisoning that are already known and some are needed to be further explored (Hill and Robinson 1945; Dresdend 1948; Keane 1972; Tschirley 1973; Longnecker et al. 1997; Conis et al. 2010; Qiu 2013). DDT was not the only culprit; other categories of synthetic pesticides such as organophosphates (OP), carbamates, and pyrethroids were also launched, and

by 1980, their impact on pest control and environment was also well recognized (Aktar et al. 2009).

OP are advanced over organochlorines being nonpersistent in environment and do not cause bioaccumulation in the food chain; however, they are much more acutely toxic to humans and other mammals than organochlorines, and their exposure by inhalation, swallowing, or absorption through the skin can lead to immediate health problems (Baird and Cann 2008). Mostly people residing in agricultural areas, farmworkers, and small children are more frequently exposed to hazards of organophosphates (Landrigan et al. 1999; Eskenazi et al. 1999; Fenske et al. 2000; McCauley et al. 2001; Quandt et al. 2004; Eskenazi et al. 2008). Acute toxicity of organophosphates and carbamates is a severe problem in developing countries, and ignorance about their hazards and the lack of information have led to many deaths among agricultural workers (Konradsen et al. 2003). A high proportion of pesticide poisonings and deaths occur in developing countries, and the main victims are farming workers especially those having insufficient knowledge of pesticide hazards (Pimentel and Greiner 1996). Intensive use of synthetic pesticides created more harsh ecological conditions that resulted in reduced soil fauna and habitat loss of micro- and macroflora (Edwards and Thompson 1973; Tripathi and Sharma 2005; Frampton et al. 2006; Bezchlebová et al. 2007). Recent studies showed that the increased use of pesticides is responsible for the vanishing population of bees and several other useful insects involved in pollination of flowers of agriculturally important crops (Gill et al. 2012). In a similar study, pesticide exposure raised question on global decline of frog population (Brühl et al. 2013). Even several species of birds have become extinct or are on the verge of it because of the pesticides. It can also be concluded that the negative impact of pesticides is much more than what is visible by the aid of the present technology.

By 2001, over 100 nations in Stockholm Convention agreed to sign an international treaty to phase out completely persistent organic pollutants ("POPs"), including DDT (Downie 2003). Albeit the synthetic pesticides have showed devastating effect on the ecosystems, in the absence of an alternative, it was impossible to diminish their utility and effects (Wu and Chen 2004; Aktar et al. 2009). Meanwhile, a phrase by Roger Ascham "Necessitie, the inuentour of all goodnesse" proved to be true for some workers in the field of biological control. Pioneer workers such as A. Bassi in Italy, V. Auduoin in France, J. Le Conte in the USA, and E. Metchnikoff in Russia proposed that antagonistic microbes might be useful in controlling crop pests and may be alternatives to synthetic pesticides (Le Conte 1874; Steinhaus 1949, 1957, 1975; McCoy et al. 1988). Later, these pioneer discoveries and further researches proved to be milestones in development of microbe-based pesticides (Schönbeck and Dehne 1986; Sundheim and Tronsmo 1988).

In the eighteenth century and even in the beginning of the nineteenth century, the focus of biological control was to use animals such as birds and entomophagous insects; microbes were not even properly known at that time. The discovery of *Bacillus thuringiensis* (Bt) showed a wider aspect of microbe-based biological control (Aronson et al. 1986; Martin and Traverse 1989; Siegel and Shadduck 1990; Marrone 1994; Joung and Coˆte' 2000). Microbial pest control was a very new concept, and its selective action on pest attracted the concentration of researchers and industrialists equally, and soon the first commercial Bt product, Thuricide, was registered in the USA in 1961 (USEPA 1998). Since then, different subspecies, varieties, and strains of Bt have been identified that are effective against a variety of insects (Gonzales et al. 1982; Carlton 1988). In a span of a very few years, Bt has covered up to 90 % of the whole biopesticide market (Chapple et al. 2000; Chattopadhyay et al. 2004; Romeis et al. 2006), and several Bt strains are now registered as biopesticides throughout the world (Glare and O'Callaghan 2000). In 1965, the first fungal product "Boverin" was developed in the former Union of Soviet Socialist Republics (USSR). In Boverin, *Beauveria bassiana* was used to control the Colorado potato beetle and the codling moth (De Faria and Wright 2007). In

1973, *Heliothis* nuclear polyhedrosis virus (NPV) was first declared as a viral biopesticide (Szewczyk et al. 2011). In the last few years, although several new biopesticides have been registered and served as better alternatives to synthetic pesticides, they still lack far behind the chemicals and the desired levels (Clemson 2007). Biopesticides have emerged as alternatives to chemical pesticides, and we also know their importance and benefits, but in reality to date, the goals have not been attained and chemicals still rule throughout the globe.

Concept of Biopesticides

In very general terms, according to the US Environmental Protection Agency (USEPA), biopesticides are pesticides derived from natural materials such as animals, plants, bacteria, and minerals. Biopesticides also include living organisms that destroy agricultural pests. The EPA separates biopesticides into three major classes based on the type of active ingredient used, namely, biochemical, plant-incorporated protectants, and microbial pesticides (USEPA 2008). Biochemical pesticides are chemicals either extracted from natural sources or synthesized to have the same structure and function as the naturally occurring chemicals. Biochemical pesticides are distinguished from conventional pesticides both by their structure (source) and mode of action (mechanism by which they kill or control pests) (O'Brien et al. 2009). At a global level, there is an inconsistency in understanding the term biopesticide as aforementioned operative definition of the term biopesticide given by USEPA is not followed in the entire world and that is why International Biocontrol Manufacturer's Association (IBMA) and the International Organization for Biological Control (IOBC 2008) promote to use the term biocontrol agents (BCAs) instead of biopesticide (Guillon 2003). IBMA classifies biocontrol agents into four groups: (1) macrobials, (2) microbials, (3) natural products, and (4) semiochemicals (insect behavior-modifying agents). Among all the biocontrol agents, the most important products are microbials (41 %), followed by macrobials (33 %), and, finally, other natural products (26 %) (Guillon 2003). This review focuses on microbe-based biopesticides.

Microbial Pesticides

Microbial pesticides are also known as BCAs. They offer the advantages of higher selectivity and lower or no toxicity in comparison to conventional chemical pesticides (MacGregor 2006). The active ingredient of a microbial pesticide is typically the microorganism. Microbial ingredients can be either the spores or the organisms themselves. The most commonly used microbial biopesticides are living organisms, which are pathogenic for the pest of interest. These include biofungicides (*Trichoderma*, *Pseudomonas*, *Bacillus*), bioherbicides (*Phytophthora*), and bioinsecticides (*Bt*) (Gupta and Dikshit 2010). Microbial pesticides come from naturally occurring or genetically altered bacteria, fungi, algae, viruses, or protozoans. They suppress pests either by producing toxic metabolites specific to the pest, causing disease, preventing establishment of other microorganisms through competition, or various other modes of action (Clemson 2007). Of the total biopesticide market for all crop types, bacterial biopesticides claim about 74 %; fungal biopesticides, about 10 %; viral biopesticides, 5 %; predator biopesticides, 8 %; and "other" biopesticides, 3 % (Thakore 2006). By 2008, there were approximately 73 microbial active ingredients that were registered by the USEPA. The registered microbial biopesticides included 35 bacterial products, 15 fungi, 6 nonviable (genetically engineered) microbial pesticides, 8 plant-incorporated protectants, 1 protozoan, 1 yeast, and 6 viruses (Steinwand 2008). Microbial biopesticides may be delivered to crops in many forms including live organisms, dead organisms, and spores, and the next subsection presents various forms of microbe-based pesticides that are being used presently.

Bacterial Biopesticides

Bacterial biopesticides are the most common form of microbial pesticides that function in multiple ways. Generally, they are used as insecticides, although they can be used to control the growth of plant pathogenic bacteria and fungi. As an insecticide, they are generally specific to individual species of moths and butterflies or species of beetles, flies, and mosquitoes. To be effective, they must come into contact with the target pest and may be required to be ingested. In insects, bacteria disrupt the digestive system by producing endotoxins that are often specific to the particular insect pest. When used to control pathogenic bacteria or fungus, the bacterial biopesticide colonizes on the plant and crowds out the pathogenic species (O'Brien et al. 2009).

The members of the genus *Bacillus* are often considered as microbial factories for the production of vast array of biologically active molecules, some of which are potentially inhibitory for fungal growth (Schallmey et al. 2004). The most widely used microbial pesticides are subspecies and strains of *B. thuringiensis* (Bt), accounting for approximately 90 % of the biopesticide market in the USA (Chattopadhyay et al. 2004). Since its discovery in 1901, Bt has been widely used to control insect pests important in agriculture, forestry, and medicine (Mazid and Kalita 2011). Its principal characteristic is the synthesis, during sporulation, of crystalline inclusions containing proteins known as δ endotoxins or Cry proteins, which have insecticidal properties. To date, over one hundred *B. thuringiensis*-based bioinsecticides, biopesticides, and biofungicides have been developed. Microbial pesticides containing *B. thuringiensis* var. *kurstaki* kill the caterpillar stage of a wide array of butterflies and moths. In addition, the genes that code for the insecticidal crystal proteins have been successfully transferred into different crop plants including cotton, tomato, brinjal, etc. that lead to significant economic benefits. Due to their high specificity and safety in the environment, *B. thuringiensis* and Cry proteins are efficient, safe, and sustainable alternatives to chemical pesticides for the control of insect pests (Roy et al. 2007; Kumar 2012).

Plant pathogenic fungi and oomycetes are major threats for crop and plant production. Therefore, the control of fungal diseases by *Bacillus*-based biopesticides represents an interesting opportunity for agricultural biotechnology. Indeed, several commercial products based on various *Bacillus* species such as *B. amyloliquefaciens*, *B. licheniformis*, *B. pumilus*, and *B. subtilis* have been marketed as biofungicides (Fravel 2005). These *Bacillus*-based products have been developed especially for the control of fungal diseases. A high number of reports have described the beneficial effects of several *Bacillus* species against diseases elicited by oomycetes and fungal pathogens. Some examples are the suppression of root diseases (such as avocado root rot, tomato damping off, and wheat take-all), foliar diseases (such as cucurbit and strawberry powdery mildews), and postharvest diseases (such as green, gray, and blue molds) (Cazorla et al. 2007; Pertot et al. 2008; Arrebola et al. 2010). Certain strains of *B. subtilis* are being used against a range of plant pathogens that cause damping off and soft rots (Kloepper et al. 2004; Haas and Defago 2005; Berg 2009). Apart from this *B. pumilus* QST 2808, *B. subtilis* QST GB03 are used for designing biopesticides, namely, Ballad®Plus and Kodiak®, for commercial purposes in the USA (Stewart et al. 2011).

Due to their catabolic versatility and excellent root-colonizing capability, pseudomonads are also being investigated extensively for the use in biocontrol of pathogens in agriculture (Ganeshan and Kumar 2006). They are known to enhance plant growth and yield, reduce severity of many diseases, and are considered to be among the most prolific PGPRs (Hoffland et al.1996; Wei et al.1996). Several species of *Pseudomonas* are being used for designing biopesticides that include *P. fluorescence*, *P. aeruginosa*, *P. syringae*, etc. Certain strains of *Pseudomonas aureofaciens* are being used against a range of plant pathogens including damping off and soft rots (Kloepper et al. 2004; Haas and Defago

2005; Berg 2009). The cell suspensions of pseudomonads are immobilized on certain carriers and are prepared as formulations for easy application, storage, commercialization, and field use. In India, *P. fluorescens* biopesticide is effectively being used against late blight of potato; it is available commercially under diverse brand names such as Krishi bio rahat, Krishi bio nidan, Mona, etc. Virulent cells of bacterial antagonist *P. fluorescens* are taken to prepare a biopesticide formulation that is effective against phytopathogen *Ralstonia solanacearum* (Bora and Deka 2007; Chakravarty and Kalita 2011).

P. syringae strains ESC-10 and ESC-11 were initially registered (licensed for sale and distribution) in 1995; at the end of April 2000, there were three end products containing ESC-10 and 2 end products containing ESC-11 in the USA (Bull et al. 1997). An attractive role of fluorescent pseudomonads in biological control of fungal plant pathogens has been illustrated against *Aspergillus*, *Alternaria*, *Fusarium*, *Macrophomina*, *Pythium*, *Sclerotinia*, and *Rhizoctonia* (Dunne et al. 1998; Gupta et al. 2001). Several different commercially available biopesticides in the USA that are developed from *Pseudomonas* and effective against fungal phytopathogens are Spot-Less, At-Eze, Bio-Save 10LP, and Bio-Save 11LP (Vargas 1999; Nakkeeran et al. 2005; Khalil et al. 2013). Bioformulation, biopesticides, and bioinoculants developed from fluorescent pseudomonads can serve multifaceted functions of plant growth promotion, bioremediation, and disease management (Arora et al. 2008, 2013; Khare and Arora 2011; Tewari and Arora 2013).

Certain other bacterial strains like that of *Agrobacterium radiobacter* are also used to control pests such as *Agrobacterium tumefaciens*. Other PGPRs like *Pantoea agglomerans* strain E325, *Streptomyces lydicus* WYEC 108, and *Coniothyrium minitans* strain CON/M/91-08 are also used nowadays for designing new biopesticides such as Bloomtime Biological™, Actinovate®SP, and Contans®WG (in the USA) that are proving to be boon in the field conditions (Chunxue et al. 2010).

Fungal Biopesticides

The fungal pathogens play a major role in the development of diseases on many important field and horticultural crops, resulting in severe plant yield losses (Khandelwal et al. 2012). Intensified use of fungicides has resulted in accumulation of toxic compounds potentially hazardous to human and environment and also in the buildup of resistance in the pathogens. Fungal biopesticides can be used to control insects and plant diseases including other fungi, bacteria, nematodes, and weeds. The mode of action is varied and depends on both the pesticidal fungus and the target pest. One advantage of fungal biopesticides in comparison with many of the bacterial and all of the viral biopesticides is that they do not need to be eaten to be effective. However, they are living organisms that often require a narrow range of conditions including moist soil and cool temperatures to proliferate. Biocontrol agents like *Trichoderma* are acclaimed as effective, eco-friendly, and cheap, nullifying the ill effects of chemicals. Therefore, of late, these biocontrol agents are identified to act against an array of important soil-borne plant pathogens causing serious diseases of crops (Bailey and Gilligan 2004).

Fungal biopesticides used against plant pathogens include *T. harzianum*, which is an antagonist of *Rhizoctonia*, *Pythium*, *Fusarium*, and other soil-borne pathogens (Harman 2005). *Trichoderma* is a fungal antagonist that grows into the main tissue of a disease-causing fungus and secretes enzymes that degrade the cell walls of the other fungus and then consumes the contents of the cells of the target fungus and multiplies its own spores. *Trichoderma* is one of the common fungal biocontrol agents being used worldwide for suitable management of various foliar- and soil-borne plant pathogens like *Ceratobasidium*, *Fusarium*, *Rhizoctonia*, *Macrophomina*, *Sclerotium*, *Pythium*, and *Phytophthora* spp. (Dominguesa et al. 2000; Anand and Reddy 2009). *Trichoderma viride* has proved to be very promising against soil-borne plant parasitic fungi (Khandelwal et al. 2012). A specific strain *Muscodor albus* QST 20799 is a

naturally occurring fungus originally isolated from the bark of a cinnamon tree in Honduras. When hydrated, this *M. albus* strain is reported to produce a number of volatiles, mainly alcohols, acids, and esters, which inhibit and kill certain bacteria and other organisms that cause soil-borne and postharvest diseases. Products containing QST 20799 can be used in fields, greenhouses, and warehouses (USEPA 2008).

B. bassiana (Balsamo) Vuillemin and *Metarhizium anisopliae* (Metchnikoff) Sorokin are naturally occurring entomopathogenic fungi that infect sucking pests including *Nezara viridula* (L) (green vegetable bug) and *Creontiades* sp. (green and brown mirids) (Sosa-Goméz and Moscardi 1998). Fungi have the unique ability to attack insects by penetrating through the cuticle making them ideal for the control of sucking pests. *B. bassiana* is currently registered in the USA as Mycotrol ES ® (Mycotech, Butte) and Naturalis L® (Troy Biosciences). These products are registered against sucking pests such as whitefly, aphids, thrips, mealybugs, leafhoppers, and weevils. Studies also show that *B. bassiana* is virulent against *Lygus hesperus* Knight (Hemiptera: Miridae), a major pest of alfalfa and cotton in the USA (Noma and Strickler 2000).

Viral Biopesticides

Like bacteria and fungi, viral biopesticides play a significant role in antagonizing pathogens especially bacteria in the form of bacteriophages. Apart from it, viruses are host specific, infecting only one or a few closely related species, thus offering minimal off-target impacts (Cory and Myers 2003; England et al. 2004; Raymond et al. 2005; Hewson et al. 2011). A bacteriophage is a virus that infects bacterial cell walls. If the virus attacks bacteria that cause plant disease, it can be used as a pesticide. A large number of phage pesticides are currently used under commercial trade names and sold in the markets. Patent protection and intellectual property are important factors in the commercialization of phage pesticides. The concept of phage therapy has existed for over 90 years, and multiple companies have acquired patents and established commercial platforms for using them (Gill et al. 2007). A leading company of the USA, Omnilytics, has developed a range of phage products for the control of *Xanthomonas campestris* pv. vesicatoria, for the treatment of bacterial spot of tomatoes and peppers, and *P. syringae* pv. tomato, which is the causative agent of bacterial speck on tomatoes (Frampton et al. 2012; Schofield et al. 2012).

Baculovirus is the main virus that is commercially used for designing phage pesticides. Since the start of their commercial use, baculoviruses have been tested extensively to assess their safety in order to meet registration requirements (reviewed in Burges et al. 1980a, b; Gröner 1986; Ignoffo 1975). Baculoviruses develop in the nuclei of the host insect cells. When ingested by the host insect, infectious virus particles are liberated internally and become active. Once in the larval gut, the virus's protein overcoat quickly disintegrates, and the viral DNA proceeds to infect digestive cells. Within a few days, the host larvae are unable to digest food and so weaken and die (Thakore 2006). Baculoviruses are particularly attractive for use as biopesticides due to their high host specificity. Each virus only attacks particular species of insects, and they have been shown to have no negative impacts on plants, mammals, birds, fish, or nontarget insects (D'Amico 2007). Baculoviruses can also cause sudden and severe outbreaks within the host population for complete control of the disease (Sylvar 2008). Another major advantage of baculoviruses is that in some cases, they can replace and serve as an alternative to the antibiotics and chemical pesticides (O'Brien et al. 2009).

As of 2010, over 24 baculovirus species have been reported to be registered for use in insect pest management throughout the world (Kabaluk et al. 2010). The market share of baculoviruses is 6 % of all microbial pesticides (Quinlan and Gill 2006; Marrone 2007), and millions of hectares have been treated with registered baculovirus products over the years (Szewczyk et al. 2009; Kabaluk et al. 2010; Moscardi et al. 2011). Despite many years of use and testing against nontarget organisms, no adverse effects have ever been attributed to baculoviruses (McWilliam 2007).

Biopesticides: Global Scenario

A large number of crop pests cause about 40 % reduction in the world's crop yield (Oerke et al. 1994), and control measures adopted by using synthetic chemicals alone have remained formidable as the data suggests that approximately 5.6 billion pounds of pesticide are used worldwide and are responsible for the unbalancing of our environment (Alavanja 2009). Whereas application of biopesticides showed lesser or no toxicity to crops and is considered environment friendly, these are not globally as dominating as synthetics. Application and development trend of biopesticides has been reviewed by Leng et al. (2011). Worldwide, approximately 1,400 biopesticide products were being sold (Marrone 2007). Table 2.1 depicts a comprehensive list of commercially available biopesticides in the markets around the globe. These products are commercially successful and widely available as liquid concentrates, wettable powders, and ready-to-use dusts and granules. Among them, bacterial products are more frequently used (Fig. 2.1) especially those from Bt (Lisansky 1997). Production of Bt always remained on priority in biopesticide industry, and currently it is the main bacterium being used in agricultural pest control (Brar et al. 2006; Ali et al. 2008). Its firm position in biopesticide industry is indicated by the fact that more than 53 % of the world biopesticide market is occupied by about 200 Bt-based products (CABI 2010), and almost 50 % of this is consumed by America particularly in the USA and Canada (Guerra et al. 2001). Data on microbial biopesticide agents from Agriculture and Agri-Food Canada (Kabaluk and Gazdik 2005) and the USEPA indicate that more than 200 products are being sold in the USA, compared to only 60 comparable products in the EU. In the UK, only 5 microbial products were reported to be sold, compared to 10 in Germany and 15 each in France and the Netherlands (Chandler et al. 2008). Till 2003, the largest market shares in biopesticide belonged to North America with 44 %, followed by Europe with 20 %, Asia (13 %), Oceania (11 %), Latin America (9 %), and lowest for Africa (3 %) (Fig. 2.2). In this section, we discuss the continent-wise scenario of biopesticides.

North America

North America leads in market share of biopesticides and covers 44 % of it. In Canada alone, end-user sales of pesticides were valued at $1.4 billion in 2010, and of this, microbial pesticides represented about 0.5 % ($7.4 million), with 88 % of microbials represented by Bt ($6 million for use in forestry; $500,000 for use in agriculture), 6.7 % ($500,000) for other bacteria, 0.67 % ($50,000) for viruses, 0.67 % ($50,000) for fungi, and 4.1 % ($300,000) for nematodes (CPL Business Consultants 2010). Numerous universities across Canada are actively engaged in the research and development of biopesticides ranging from botanical to microbial pesticides for control of insects, weeds, and fungi. In the USA, the first registered microbial pesticide was prepared using *Bacillus popilliae* (*Paenibacillus popilliae*) in 1948 for the control of the Japanese beetle (Schneider 2006). Whereas a commercially unsuccessful entomopathogenic fungus (*Hirsutella thompsonii*)-based product Mycar was first registered for control of citrus rust mites, for which approval was granted to Abbott Laboratories in 1981 (McCoy 1996). In the USA, EPA is the sole agency responsible for encouraging development and use of biopesticides. Biopesticides and Pollution Prevention Division (under the Pesticide Programs) was established in 1994 in the USA with the goal of reducing risks associated with pesticide use in agricultural and nonagricultural settings and advocates adoption of biopesticides. In the USA, pesticide has to pass stringent regulations before its marketing, and the Federal Insecticide, Fungicide, and Rodenticide Act (FIFRA) does this job along with EPA, assessing carcinogenic risks or short-term mutagenicity assays to ensure that pesticide can be used with a reasonable certainty, and it will not harm human health or the environment. Such stringency in regulation created a positive pressure on growers to adopt biopesticides.

Table 2.1 Commercially available biopesticides in the global market

Category of biopesticide	Products common name or trade name	Targets
The USA		
Bactericides		
A. radiobacter k84	Galltrol – A	Crown gall disease
P. agglomerans C9-1	BlightBan C9-1	Fire blight
P. agglomerans E325	Bloomtime	Fire blight
Bacteriophage of *P. syringae* pv. Tomato	AgriPhage	Bacterial speck
Bacteriophage of *X. campestris* pv. Vesicatoria	AgriPhage	Bacterial spot
Fungicides		
B. licheniformis SB3086	EcoGuard	Fungal diseases
Bacillus mycoides isolate J	BacJ	Cercospora
B. pumilus GB 34	GB34	Seedling diseases – *Pythium* and *Rhizoctonia*
B. pumilus QST 2808	Sonata	Powdery mildew, downy mildew, and rusts
	Ballad Plus	
B. subtilis GB03	Companion	*Fusarium*, *Pythium*, *Rhizoctonia*
	Kodiak	
B. subtilis MBI 600	Histick N/T	Damping off
	Pro-Mix with Biofungicide	
B. subtilis subsp. amyloliquefaciens FZB24	Taegro	*Fusarium* and *Rhizoctonia* wilt diseases
P. aureofaciens Tx-1	Spot-Less	Turf fungal diseases
Pseudomonas chlororaphis 63–28	At-Eze	Soil and seed-borne fungi
P. syringae ESC 10	Bio-Save 10LP	Postharvest diseases
P. syringae ESC 11	Bio-Save 11LP	Postharvest diseases
Streptomyces griseoviridis K61	Mycostop	Fungi causing damping off, stem, and crown rots
	Biofungicide	
	Mycostop Mix	
S. lydicus WYEC108	Actinovate	Fungi causing damping off, stem and crown rots
	Actino-Iron	
Ampelomyces quisqualis M10	PowderyGon	Powdery mildew
Aspergillus flavus AF36	*Aspergillus flavus* AF36	*Aspergillus flavus* producing aflatoxin
A. flavus NRRL 21882	Afla-guard	*Aspergillus flavus producing aflatoxin*
C. minitans CON/M/91–08	Contans	*Sclerotinia minor*, *Sclerotinia sclerotiorum*
Gliocladium catenulatum J1446	Prestop	Seed-borne and soil-borne diseases
M. albus QST 20799	Arabesque	Postharvest diseases
Pseudozyma flocculosa PF-A22 UL	Sporodex	Powdery mildew
Trichoderma asperellum ICC 012 and *T. harzianum* (*gamsii*) ATCC080	Tenet	Soil-borne diseases
	Bioten	
	Remedier	
T. harzianum ATCC 20476	Binab	Wound healing
T. harzianum Rifai T-22	PlantShield	Seed and foliar diseases
	RootShield	
	T-22 Planter box	
T. harzianum T-39	Trichodex	Soil and foliar diseases

(continued)

Table 2.1 (continued)

Category of biopesticide	Products common name or trade name	Targets
Trichoderma polysporum ATCC 20475	Binab T	Soil and foliar diseases
Ulocladium oudemansii U3	BOTRY-Zen	Botrytis and *Sclerotinia*
Verticillium albo-atrum WC S850	DutchTrig	Dutch elm disease
Bacteriophage of *P. syringae* pv. tomato	AgriPhage	Tomato leaf spot
Candida oleophila strain O	NEXY	Postharvest fruit molds
Fungicides/bactericides		
B. subtilis QST713	Serenade	Foliar fungal and bacterial diseases
Herbicides		
Bacillus cereus BP01	MepPlus	Plant growth regulator
Alternaria destruens 059	Smolder	Herbicide – dodder
Chondrostereum purpureum PFC 2139	Chontrol Paste	Herbicide – stump sprout inhibitor
Colletotrichum gloeosporioides f.sp. aeschynomene ATCC 202358	LockDown	Herbicide – northern Jointvetch
Puccinia thlaspeos woad (dyer's woad rust)	Woad Warrior	Herbicide – Dyer's woad
Insecticides		
B. popilliae	Milky Spore Powder	Japanese beetle grubs
Bacillus sphaericus Serotype H5a5b strain 2362 ATCC1170	VectoLex	Mosquito larvae
B. thuringiensis subsp. aizawai NB200	Florbac	Moth larvae
B. thuringiensis subsp. israelensis	BMP	Mosquito and blackflies
B. thuringiensis subsp. israelensis EG2215	Gnatrol	Mosquito, flies
	Aquabac	
B. thuringiensis subsp. aizawai delta-endotoxin in killed *P. fluorescens*	M-Trak	Colorado potato beetle
B. thuringiensis subsp. aizawai GC-91	Agree WG	Plutella
B. thuringiensis subsp. kurstaki	Thuricide Forestry	Lepidopteran larvae
	Wilbur-Ellis BT 320	
	Dust	
	Dipel	
	Deliver	
	Biobit HP	
	Foray	
	Javelin WG	
	Green Light	
	Hi-Yield Worm Spray	
	Ferti-Lome	
	Bonide	
	Britz BT	
	Worm Whipper	
	Security Dipel Dust	
B. thuringiensis subsp. kurstaki BMP 123	BMP123	Lepidopteran larvae
B. thuringiensis subsp. kurstaki EG2348	Condor	Lepidopteran larvae
B. thuringiensis subsp. tenebrionis	Novodor	Colorado potato beetle

(continued)

Table 2.1 (continued)

Category of biopesticide	Products common name or trade name	Targets
B. thuringiensis subsp. kurstaki EG7826	Lepinox WDG	Lepidopteran larvae
B. bassiana 447	Baits Motel Stay-awhile	Ants
B. bassiana ATCC 74040	Naturalis L	Various insects
B. bassiana GHA	Mycotrol ES	Various insects
	Mycotrol O	
	Botanigard 22WP	
	BotaniGard ES	
B. bassiana HF23	balEnce	Housefly
M. anisopliae F52	Tick-Ex	Ticks and grubs
Paecilomyces fumosoroseus Apopka 97	PFR-97	Whitefly and thrips
Nosema locustae	Nolo-Bait	Grasshopper and crickets
	Semaspore Bait	
Anagrapha falcifera NPV	CLV-LC	Lepidopteran larvae
Cydia pomonella GV	CYD-X	Virus codling moth
Gypsy moth NPV	Gypchek	Gypsy moth
H. zea NPV (previously *Heliothis zea* NPV)	GemStar	Cotton bollworm, tobacco, budworm
Indian meal moth GV (*Plodia interpunctella* GV)	FruitGuard	Indian meal moth
Mamestra configurata NPV (107308)	Virosoft	Bertha armyworm
Spodoptera exigua NPV	Virus Spod-X	Beet armyworm
Saccharomyces cerevisiae	Bull Run	Fly attractant
Nematicides		
Bacillus firmus I-1582	BioNem	Nematodes
Pasteuria usgae	Econem	Nematodes
Myrothecium verrucaria	DiTera	Nematodes
Paecilomyces lilacinus 251	MeloCon WG	Nematodes
Virucides		
Zucchini yellow mosaic virus – weak strain	AgroGuard-Z	Zucchini yellow mosaic
Europe		
Aureobasidium pullulans	Blossom Protect	Fire blight, postharvest diseases
Phlebiopsis gigantea (several strains)	Rotstop	Conifer root rots
P. chlororaphis	Cedomon, Cerall	*Pyrenophora teres*, *P. graminea*, *Tilletia caries*, *Septoria nodorum*, *Fusarium* spp.
Pseudomonas sp. DSMZ 13134	Proradix	Root rots
S. griseoviridis K61	Mycostop	*Fusarium* wilt, *Botrytis* gray mold, root rot, stem rot, stem end rot, damping off, seed rot, soil-borne damping off, crown rot, *Rhizoctonia*, *Phytophthora*, wilt, seed damping off, early root rot
A. quisqualis AQ10	AQ10	*Leaf disease*
C. oleophila strain O		Postharvest disease
C. minitans CON/M-91–05	Contans WG	*Sclerotinia sclerotiorum*, *S. minor*
G. catenulatum J1446	Prestop, Prestop mix	Damping off, gummy stem blight, gray mold, root rot, stem rot, wilt, storage diseases, foliar diseases, seed rot

(continued)

Table 2.1 (continued)

Category of biopesticide	Products common name or trade name	Targets
P. flocculosa PF-A22 UL	Sporodex	Powdery mildew
Pythium oligandrum	Polyversum	Root rots
Trichoderma asperellum (ICC012) (T25) (TV1) (formerly *T. harzianum*)	Tenet	Fungal infections (*Pythium*, *Phytophthora*, *Botrytis*, *Rhizoctonia*)
Trichoderma atroviride IMI 206040 (formerly *T. harzianum*)	Binab T Pellets	*Botrytis cinerea*, pruning wound infection *Chondrostereum purpureum*
T. atroviride I-1237	Esquive	Fungal infections (*Pythium*, *Phytophthora*, *Botrytis*, *Rhizoctonia*)
Trichoderma gamsii (formerly *T. viride*) (ICC080)	Remedier	Fungal infections (*Pythium*, *Phytophthora*, *Botrytis*, *Rhizoctonia*)
T. harzianum Rifai T-22 ITEM 108 or KRL-AG2	Trianum P	Root diseases
T. harzianum Rifai T-39 (IMI 206039)	Trichodex	*Botrytis cinerea*, *Colletotrichum* spp., *Fulvia fulva*, *Monilia laxa*, *Plasmopara viticola*, *Pseudoperonospora cubensis*, *Rhizopus stolonifer*, *Sclerotinia sclerotiorum*
	Rootshield	
T. polysporum and *T. harzianum*	Binab T Vector	Fungal pathogens, fairy ring, *Botrytis*, *Verticillium*, *Pythium*, *Fusarium*, *Phytophthora*, *Rhizoctonia*, *Didymella*, *Chondrostereum*, *Heterobasidion*
V. albo-atrum (WCS850) (formerly *Verticillium dahliae*)	Dutch Trig	Dutch elm disease
Fungicides/bactericides		
B. subtilis QST 713	Serenade	Fire blight, *Botrytis* spp.
Insecticides		
B. thuringiensis subsp. *aizawai* GC-91	Turex	Lepidoptera pests
B. thuringiensis subsp. *israelensis* AM65	VectoBac	Sciarids
B. thuringiensis subsp. *kurstaki* HD-1	Dipel WP	Lepidoptera pests
B. thuringiensis subsp. kurstaki ABTS 351, PB 54, SA 11, SA12, and EG 2348	Batik	Lepidoptera pests
	Delfin	
B. thuringiensis subsp. *kurstaki* BMP 123	BMP 123	Lepidoptera pests
	Prolong	
B. thuringiensis subsp. *tenebrionis* NB 176	Novodor	Coleoptera pests
B. bassiana ATCC 74040	Naturalis L	Thrips, whitefly, mites
B. bassiana GHA Fungus	Botanigard	Whiteflies, aphids, thrip
Lecanicillium muscarium (Ve6) (former *Verticillium lecanii*)	Mycotal, Vertalec	Whiteflies, thrips, aphids (except the Chrysanthemum aphid: *Macrosiphoniella sanborni*)
P. fumosoroseus Apopka 97	Preferal WG	Greenhouse whiteflies (*Trialeurodes vaporariorum*)
P. fumosoroseus Fe9901	Nofly	Whiteflies
Adoxophyes orana BV-0001 GV	Capex	Summer fruit tortrix (*Adoxophyes orana*)
Cydia pomonella GV	BioTepp	Codling moth (*Cydia pomonella*)
Spodoptera exigua NPV	Spod-X GH	*Spodoptera exigua*

(continued)

Table 2.1 (continued)

Category of biopesticide	Products common name or trade name	Targets
Nematicides		
P. lilacinus PL 251	BioAct WG	Common plant parasitic nematodes
Virucides		
Zucchini Yellow Mosaic Virus, weak strain Virus	Curbit	Yellow mosaic virus
China		
Bactericides		
A. radiobacter	Trade name not available	Crown gall
Bacillus polymyxa	Trade name not available	Crown gall
Bacillus sphaericus	Trade name not available	Crown gall
Fungicides		
B. cereus	Trade name not available	Bacterial wilt, sheath blight/rice false smut, bacterial wilt
B. licheniformis		Downy mildew, *Fusarium* wilt
B. subtilis	Trade name not available	Bacterial wilt, root rot, tobacco black shank, rice blast, rice false smut
Trichoderma spp.		Fungus downy mildew, *Rhizoctonia cerealis*, gray mold
Fungicides/bactericides		
P. fluorescens	Trade name not available	Bacterial wilt, root rot
Insecticides		
B. thuringiensis subsp. aizawa	Trade name not available	Lepidopteran pests
B. thuringiensis subsp. israelensis	Trade name not available	Lepidopteran pests
B. thuringiensis subsp. kurstaki		Lepidopteran pests
Pseudomonas alcaligenes	Trade name not available	Locusts, grasshoppers
B. bassiana		*Monochamus alternatus*, *Dendrolimus punctatus*
Conidobolus thromboides	Trade name not available	Aphids
M. anisopliae		Cockroaches, grasshoppers, locusts
P. lilacinus	Trade name not available	Nematodes
Pochonia chlamydosporia		Nematodes
Dendrolimus cytoplasmic polyhedrosis virus	Trade name not available	Virus Caterpillars
NPV, *Ectropis obliqua hypulina* NPV, *Laphygma exigua* NPV, *Prodenia litura* NPV, *Buzura suppressaria* NPV, *Gynaephora ruoergensis* NPV, *Mythimna separata* NPV	Trade name not available	Virus Beet armyworm, lepidoptera, looper, *H. armigera*, *Laphygma exigua*
Periplaneta fuliginosa densovirus virus	Trade name not available	Cockroaches
Pieris rapae GV, *Mythimna separata* GV, *Plutella xylostella* GV	Trade name not available	*Pieris rapae*, *Plutella xylostella*
Japan		
Insecticides (bacterial, fungal, viral, parasitic nematodes)		
B. thuringiensis kurstaki	Toarowaa Esmark Guardjet, Dipol, Tuneup Fivestar BioMax DF	Lepidopteran larvae

(continued)

Table 2.1 (continued)

Category of biopesticide	Products common name or trade name	Targets
B. thuringiensis aizawai	Quark XenTari Florbac Sabrina	Lepidopteran larvae
B. thuringiensis aizawai +kurstaki	Bacilex	Lepidopteran larvae
B. thuringiensis japonensis	BuiHunter	Cockchafers and white grubs
B. bassiana	BotaniGard	Thrips, whiteflies, diamondback moth
P. fumosoroseus	Preferd	Whitefly, aphids
Lecanicillum longisporum	Vertalec	Aphids
Adoxophyes orana GV+Homona magnanima GV	Hamaki-Tenteki	*Adoxophyes honmai* and *Homona magnanima*
Steinernema carpocapsae	Bio Safe	Weevils, black cutworm, common cutworm, peach fruit moth
India		
Fungicide		
P. fluorescens	ABTEC Pseudo	Plant soil-borne diseases
	Biomonas	
	Esvin Pseudo	
	Sudo	
	Phalada 104PF	
	Sun Agro Monus	
	Bio-cure-B	
A. quisqualis	Bio-Dewcon	Powdery mildew
T. harzianum	Biozim	Soil-borne pathogens
	Phalada 105	
	Sun Agro Derma H	
T. viride	Monitor, Trichoguard	Soil-borne pathogens
	NIPROT	
	Bioderma	
	Biovidi	
	Eswin Tricho	
	Biohit	
	Tricontrol	
	Ecoderm	
	Phalada 106TV	
	Sun Agro Derma	
	Defense SF	
Fungicides/bactericides		
B. subtilis		Soil-borne pathogens
Insecticides		
B. thuringiensis subsp. israelensis	Tacibio, Technar	Lepidopteran pests
B. thuringiensis subsp. Kurstaki	Bio-Dart	Lepidopteran pests
	Biolep	
	Halt	
	Taciobio-Btk	

(continued)

Table 2.1 (continued)

Category of biopesticide	Products common name or trade name	Targets
B. bassiana	Myco-Jaal	Coffee berry borer, diamondback moth, thrips, grasshoppers, whiteflies, aphids, codling moth
	Biosoft	
	ATEC Beauveria	
	Larvo-Guard	
	Biorin	
	Biolarvex	
	Biogrubex	
	Biowonder	
	Veera	
	Phalada 101B	
	Bioguard	
	Bio-power	
M. anisopliae	ABTEC	Coleoptera and lepidoptera, termites, mosquitoes, leafhoppers, beetles, grubs
	Verticillium	
	Meta-Guard	
	Biomet	
	Biomagic	
	Meta	
	Biomet	
	Sun Agro Meta	
	Bio-Magic	
P. fumosoroseus	Nemato-Guard	Whitefly
	Priority	
P. lilacinus	Yorker	Whitefly
	ABTEC	
	Paceilomyces	
	Paecil	
	Pacihit	
	ROM biomite	
	Bio-Nematon	
Verticillium lecanii	Verisoft	Whitefly, coffee green bug, homopteran pests
	ABTEC	
	Verticillium	
	Vert-Guard	
	Bioline	
	Biosappex	
	Versitile	
	Ecocil	
	Phalada 107 V	
	Biovert Rich	
	ROM Verlac	
	ROM Gurbkill	
	Sun Agro Verti	
	Bio-Catch	

(continued)

Table 2.1 (continued)

Category of biopesticide	Products common name or trade name	Targets
H. armigera NPV	Helicide	*H. armigera*
	Virin-H	
	Helocide	
	Biovirus-H	
	Helicop	
	Heligard	
Spodoptera litura NPV	Spodocide	*S. litura*
	Spodoterin	
	Spodi-cide	
	Biovirus-S	
Nematicides		
Verticillium chlamydosporium		Nematodes
Australia		
Fungicide		
P. fluorescens	ABTEC Pseudo	Plant soil-borne diseases
	Biomonas	
	Esvin Pseudo	
	Sudo	
	Phalada 104PF	
	Sun Agro Monus	
	Bio-cure-B	
A. quisqualis	Bio-Dewcon	Powdery mildew
T. harzianum	Biozim	Soil-borne pathogens
	Phalada 105	
	Sun Agro Derma H	
T. viride	Monitor, Trichoguard	Soil-borne pathogens
	NIPROT	
	Bioderma	
	Biovidi	
	Eswin Tricho	
	Biohit	
	Tricontrol	
	Ecoderm	
	Phalada 106TV	
	Sun Agro Derma	
	Defense SF	
Fungicides/bactericides		
B. subtilis		Soil-borne pathogens
Insecticides		
B. thuringiensis subsp. israelensis	Tacibio, Technar	Lepidopteran pests
B. thuringiensis subsp. Kurstaki	Bio-Dart	Lepidopteran pests
	Biolep	
	Halt	
	Taciobio-Btk	

(continued)

Table 2.1 (continued)

Category of biopesticide	Products common name or trade name	Targets
B. bassiana	Myco-Jaal	Coffee berry borer, diamondback moth, thrips, grasshoppers, whiteflies, aphids, codling moth
	Biosoft	
	ATEC Beauveria	
	Larvo-Guard	
	Biorin	
	Biolarvex	
	Biogrubex	
	Biowonder	
	Veera	
	Phalada 101B	
	Bioguard	
	Bio-power	
M. anisopliae	ABTEC	Coleoptera and lepidoptera, termites, mosquitoes, leafhoppers, beetles, grubs
	Verticillium	
	Meta-Guard	
	Biomet	
	Biomagic	
	Meta	
	Biomet	
	Sun Agro Meta	
	Bio-Magic	
P. fumosoroseus	Nemato-Guard	Whitefly
	Priority	
P. lilacinus	Yorker	Whitefly
	ABTEC	
	Paceilomyces	
	Paecil	
	Pacihit	
	ROM biomite	
	Bio-Nematon	
V. lecanii	Verisoft	Whitefly, coffee green bug, homopteran pests
	ABTEC	
	Verticillium	
	Vert-Guard	
	Bioline	
	Biosappex	
	Versitile	
	Ecocil	
	Phalada 107 V	
	Biovert Rich	
	ROM Verlac	
	ROM Gurbkill	
	Sun Agro Verti	
	Bio-Catch	

(continued)

Table 2.1 (continued)

Category of biopesticide	Products common name or trade name	Targets
H. armigera NPV	Helicide	*H. armigera*
	Virin-H	
	Helocide	
	Biovirus-H	
	Helicop	
	Heligard	
S. litura NPV	Spodocide	*S. litura*
	Spodoterin	
	Spodi-cide	
	Biovirus-S	
Nematicides		
Verticillium chlamydosporium		Nematodes
Bactericides		
A. radiobacter	NoGall	Crown gall disease
Fungicides		
T. harzianum	Trichodex	*Botrytis* spp.
Insecticides		
B. sphaericus	VectoLex	Mosquito larvae
B. thuringiensis subsp. a*izawai*	Agree, Bacchus, XenTari	Lepidoptera larvae
B. thuringiensis subsp. israelensis	Aquabac, BTI, Teknar, Vectobac	Mosquito larvae
B. thuringiensis subsp. kurstaki	Biocrystal, Caterpillar, Killer, DiPel, Costar, Delfin, Full-Bac WDG	Lepidoptera larvae
M. anisopliae	BioCane, Granules	Gray-backed cane grub (scarabs)
M. anisopliae subsp. *acridum*	Green Guard	Locusts and grasshoppers
M. flavoviride	Chafer Guard	Redheaded pasture cockchafer
H. armigera NPV	Heliocide	*Helicoverpa* spp.
	Vivus Gold	
	Vivus Max	
H. zea NPV	Gemstar	*Helicoverpa* spp.
	Vivus	
Africa		
Bactericides	**Products**	**Targets**
A. radiobacter	Crown Gall Inoculant	Crown gall
Fungicides		
B. subtilis 101	Shelter	Root and leaf diseases
B. subtilis 102	Artemis	Root and leaf diseases
B. subtilis 246	Avogreen	Root and leaf diseases
B. subtilis QST 713	Serenade	*Botrytis* spp.
A. quisqualis AQ10	Bio-Dewcon	Powdery mildew

(continued)

Table 2.1 (continued)

Category of biopesticide	Products common name or trade name	Targets
T. harzianum	Eco-77	Root diseases
	Eco-T	
	Promot	
	Romulus	
	Rootgard	
	Trichoplus	
	Trykocide	
T. harzianum 39	Trichodex	Root diseases
T. harzianum DB103	T-Gro	Root diseases
Fungicides/bactericides		
B. subtilis	Defender	Soil-borne fungi and bacteria
Insecticides		
B. thuringiensis subspp. aizawai and kurstaki	Agree	Lepidoptera larvae
B. thuringiensis subsp. israelensis	VectoBac	Mosquito
B. thuringiensis subsp. kurstaki	DiPel	Lepidoptera larvae
	Rokur	
	Thuricide	
B. thuringiensis subsp. kurstaki H7	Florbac WG	Lepidoptera larvae
B. bassiana	Bb Plus, Bb weevil, Sparticus	Thrips, weevils, whiteflies
M. anisopliae subsp. acridum IMI 330 189	Green Muscle	Locust
GV	Trade name not available	Lepidoptera larvae
Pseudomonas resinovorans bacteriophage	Agriphage	Insect pest control
Nematicides		
P. lilacinus	Bio-Nematon	Nematodes
P. lilacinus 251	PL Plus	Nematodes

Source: Kunimi (2007) and Kabaluk et al. (2010)

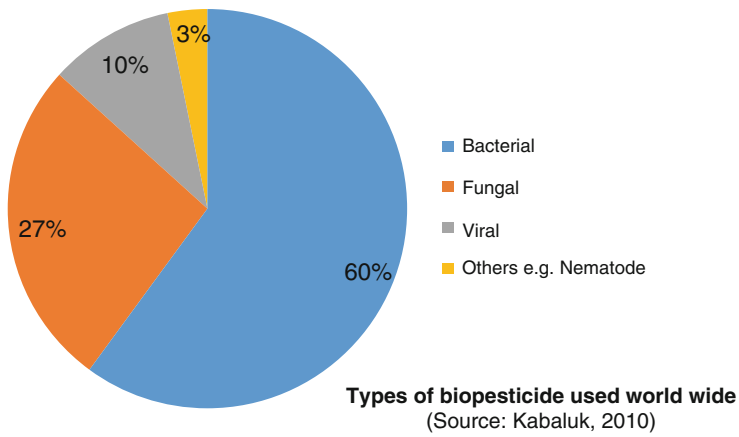

Fig. 2.1 Global biopesticide market based on types of microbes used

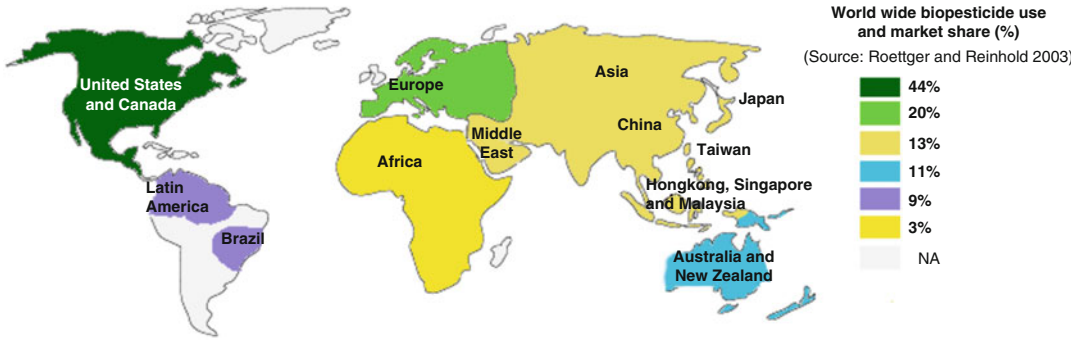

Fig. 2.2 Global biopesticide use and market

Keeping in mind the importance of minor crops (defined as any crop grown on 300,000 acres or less), the US Department of Agriculture (USDA) initiated the Interregional Research Project No. 4, commonly known as IR-4. The USDA IR-4 program has provided grant funding to key influencers such as land-grant university extension specialists to demonstrate with the end user the performance of biopesticides in realistic programs with conventional pesticides (Radcliffe et al. 2009). Overall progress of biopesticides in the USA can be considered as satisfactory. According to EPA data in the USA, 102 microbials, 52 biochemicals, and 48 semiochemicals are being used as biopesticides (USEPA 2011).

Europe

Europe ranks second in biopesticide production. The largest individual European biopesticide market is Spain, followed by Italy and France (Business Wire 2010). The first Bt-based product (Thuricide) was approved in Europe in 1964, whereas first registration for an entomopathogenic fungus *L. longisporum* was given in 1981 to Tate and Lyle in the UK (Quinlan 1990). Europe also belongs to continents where the MRL (Maximum Residue Level) regulations in food products have been strictly decided (Regulation (EC) 396/2005), and the European Food Safety Authority (EFSA) has an important role in this system. EFSA provides independent scientific advice to support the risk managers (EU institutions and Member States) in defining appropriate regulatory frameworks and making decisions to protect consumers. These may involve adopting or revising European legislation on food or feed safety or deciding whether to approve regulated substances such as pesticides and food additives, and if so in which foods or crops and at what levels. This resulted in farmers to produce crop having very less concentration of pesticide or with no detectable limit. In this residue-controlling movement the Ministry of Food, Agriculture, and Fisheries in France launched the program "Ecophyto 2018," in 2008, for a 50 % reduction in the use of pesticides by 2018 (agriculture.gouv.fr/IMG/pdf/PLAN_ECOPHYTO_2018_eng.pdf). In 2011, Europe implemented the Plant Protection Products Regulation (www.eppo.int/PPPRODUCTS/information/new_eu_regulations.htm), and according to that, any crop protection product validates not only that it is effective but also that it should be risk free to humans and to the environment. This made 74 % of all commercial pesticides illegal, as only 26 % of substances passed the new stringent tests, biopesticides being among them (www.pangaeaventures.com/blog/biopesticides-the-next-crop-of-cleantech-home-runs). Currently, most of the European consumers are demanding production of food that hasn't been treated with conventional pesticides; that is why in the present time, Europe has set the standard for biopesticides use. In spite of the stark law on residue control, development of new biopesticide faces hurdle of registration which may take at least 5 years and cost up to € 0.5 million. Presently there are 68 biopesticide active substances registered in

Europe that consist of 34 microbials, 11 biochemicals, and 23 semiochemicals (EUPD 2010). The proportion of the European microbial pesticide market taken by *B. thuringiensis*-based products has declined from an estimated 90 % in 2000 to 50.6 % in 2008 mainly because of the arrival of other BCAs (Business Wire 2010). The largest increases since 2005 were seen in non-Bt bacteria, notably *B. subtilis*, and in fungal-based products, including *C. minitans* and *Trichoderma*. There were also significant increase in viral sales and a steady rise in the nematode market (Business Wire 2010).

Asia

Asia is the largest continent in the world and known for its diverse biodiversity. In Asia, the economy depends heavily on agriculture. Using biopesticides in food crops such as rice, maize, and vegetables is increasing gradually. Rice is the main staple hence much of the biocontrol experimentation and practical use was done on this crop. China is one of the most populated countries in the world, and it is producing biopesticides since 1960, most of them being in the form of unformulated dried cultures (Xu et al. 1987). Biopesticide application was done in 800,000 ha in 1972 which reached 27,000,000 ha in 2000 (Ye and Chen 2002; Zhang 2002; Yang 2007). Till 2008, there were 327 biopesticides registered in China. Among these, 270 bacterial biopesticides are obtained from 11 microbial species (mostly Bt), 22 registered fungal biopesticides from 6 fungal species, and 35 registered viral biopesticides, 14 of which are developed from *Heliothis armigera* NPV (ICAMA 2008).

Japan is one of the pioneer countries as far as the use of biopesticides is considered. In Japan, registration process for Bt was a typical case (Aizawa and Ishiwata 2001) as the earlier application had posed a negative impact on sericulture. In 1972, a study committee on Bt products was established for studying the effects of Bt products on silkworm rearing in sericulture (Aizawa and Fujiyoshi 1973), and after 7 years, the committee concluded that Bt products would not pose a threat to silkworm rearing if farmers were prevented from spraying Bt products on mulberry fields (Study Committee on *Bacillus thuringiensis* Products 1984). Finally, the first Bt registered product was launched in 1981 (Kunimi 2007) and later formulated products of Bt aizawai, Bt kurstaki, and Bt japonensis were also introduced (Ohba et al. 1992). NPV was first time used to control cabbage armyworm, *Mamestra brassicae*, in 1962 (Kunimi 1998). Entomopathogenic fungus *B. bassiana* was also tried to control pine caterpillar (*Dendrolimus spectabilis*) in 1933 by Hidaka (1933). In the last few years, Japanese research in biocontrol field has resulted in the identification and characterization of several new insect pathogens, delivery systems, and formulation development, but the sale of microbial-based products remained less satisfactory, even less than 2 % of all insecticides sold (Kunimi 2007).

In India, biocontrol concept was in practice ever since neem was used as an alternative to chemical pesticides. Farmers have been using neem not only for vegetable protection but also in various other medically imported applications. Evidence of using insects and birds for pest eradication is also found (Subramaniam 1952). In India, microbial-based pesticides evolved as an emergent need when chemical insecticides failed to control *Helicoverpa armigera*, *S. litura*, and other pests of cotton (Armes et al. 1992; Kranthi et al. 2002). In India, the first time commercial production of biocontrol agents was started by Bio-Control Research Laboratories (BCRL), a division of Pest Control (India) Limited, under contract with Plant Protection Research Institute (PPRI) (Manjunath et al. 1992). The rise of biopesticides in India is being encouraged by the government as part of the integrated pest management (IPM) program. The Ministry of Agriculture and the Department of Biotechnology are largely responsible for supporting the production and application of biopesticides, and most of biopesticides are being supplied free of cost by the research agencies to farmers through extension services (Alam 1994). In the last few years, microbes exhibiting good biocontrol potential have been discovered

by many workers and are commercially exploited for large-scale biopesticide development (Rabindra 2001; Ignacimuthu et al. 2001; Koul et al. 2003; Ranga Rao et al. 2007). Most of the products are from antagonistic fungi (especially *Trichoderma* spp.) and bacteria (especially Bt and *P. fluorescens*), whereas viral biopesticides consist of NPV and granuloviruses (GV) (Rabindra 2005). But large-scale production poses certain difficulties, and lesser developed technology is the main hurdle for industrial production. Hence, most of the products are being produced at small-scale facilities for local use only, mostly by sugar mills and cooperatives, state agricultural departments, IPM centers, and agricultural universities. By 2006, only 12 biopesticides (that of Bt, *Trichoderma*, *Pseudomonas*, and *Beauveria* species) had been registered, but 194 substances were listed as chemical pesticides (Gupta 2006). However, biopesticides consumption has increased in India as there was 219 metric tons (MT) of biopesticide used in 1996–1997 which increased to 683 MT in 2000–2001, whereas load of chemical pesticides had declined from 56,114 MT to 43,584 MT in the same years (Shukla and Shukla 2012). India is still facing problems associated with production, and quality of the products are not up to the desired level because of which farmers are not very enthusiastic for biopesticides in comparison to chemical pesticides (Gupta and Dikshit 2010).

Bt was the first biopesticide product to be introduced and commercialized into the Thai market in 1965 (Rushtapakomchai 2003). But it was largely neglected by farmers as they did not know much about Bt due to its slow and highly selective action (Rushtapakomchai 2003). Finally in 1969, Bt kurstaki was introduced to control lepidopterous larvae of cruciferous crops (Prasetphol et al. 1969; Vattanatangum 1989). Active research on biopesticide started in 1980 to produce products containing Bt, NPV, and fungi (Jones et al. 1993). The promotion of biopesticide is going on in Thailand, and by 2003 there were six major biopesticides in the market from Bt (subspecies kurstaki, aizawai, and tenebrionis), fungal products of *T. harzianum*, entomopathogenic fungi, entomopathogenic nematodes, and NPV (Rushtapakomchai 2003). Although the production is increasing, it is not enough to fulfill the demand of the whole country, whereas poor quality of some of the noncommercial biopesticides is also a cause for concern (Warburton et al. 2002).

In South Korea, microbial pest control was initiated in 1970 and involved the use of entomopathogenic viruses, bacteria, fungi, and nematodes to control pests in forestry, agriculture, and golf courses, but it took many years when the first commercial biopesticide, "Solbichae" (a Bt subsp. aizawai), was registered in 2003 to control diamondback moth and beetle armyworm in Chinese cabbage (Jeong et al. 2010). By 2009, 34 microbial pesticide products were registered to control insect pests and plant diseases in Korea (Jeong et al. 2010).

Australia

Microbial control first began in the late 1960s with the GV of codling moth (*Cydia pomonella*) and nucleopolyhedrosis virus (NPV) of *Helicoverpa zea*. Initially, limited success was observed in field trials in comparison with their chemical counterparts, but GV proved to be effective against potato tuber moth (*Phthorimaea operculella*) (Reeda and Springetta 1971). A vast variety of products have been registered, but among them *B. thuringiensis* subsp. *kurstaki* (Btk) is the most famous product, and in 1987–1988 when there was increased incidence of cotton pest, Btk-based products accounted for a 2 % market share of all the insecticides (Powles and Rogers 1989; Fitt 1994, 2004). Fungal biocontrol research was mainly directed toward *Metarhizium* to control a wide range of insects (Milner and Jenkins 1996) but especially its effectiveness against locust and grasshopper was of much interest and its oil-based formulation with high persistence in soil made it very popular in Australia (Ibrahim et al. 1999; Milner et al. 1997; Lomer 2001). BioGreen was first registered and commercially produced *Metarhizium*-based product. After the success of BioGreen, other *Metarhizium*-based products also came up and

showed remarkable response in biocontrol of crop diseases (Milner 2000).

Latin America

There is a growing trend of using biopesticides in organic culture in Latin America, especially at the local level production for indigenous use has increased meaningfully by the involvement of the local government and NGOs. In Latin America, a proportion of Bt-based products cover about 40 % of the market (CPL Business Consultants 2010). In Argentina, Bt products were first used in 1950 against *Colias lesbia* in alfalfa (Botto 1996). The first virus-based product was registered in 2000 by Agro Roca, and the fungal product based on *B. bassiana* for controlling *Triatoma infestans* and *Musca domestica* was first registered by Alves et al. (2008). The use of biopesticides has risen in Brazil, and by 2010 approximately 3 million hectares of agricultural cropland were being treated annually with microbial pesticides (Kabaluk et al. 2010). The use of Bt in Brazil started in the early 1990s, and at that time only three commercial products were available in the Brazilian market, all based on Btk (Dipel, Thuricide, and Bactospeine) (Habib and Andrade 1991). Approximately 40 commercial mycoinsecticides available in the Brazilian market are registered by 19 for-profit companies. More than 20 laboratories operated by sugar/ethanol mills produce *M. anisopliae* for their own use to control cercopids in cane fields (Kabaluk et al. 2010). Universities, research institutes, nonprofit organizations, rubber tree farms, and cattle farms also produce various fungal microbial control agents. By 2011, there were only 26 BCAs registered that is far less in comparison to 1,352 chemical pesticides (formulations and mixtures) registered. However, at the same time a number of unregistered BCA are also sold, and their number is higher in comparison to registered products, as the registration process involves high cost and length of time (Bettiol 2011). However, Argentina and Brazil have showed potential in utilizing biocontrol agents, and Cuba is also one of the leads in the pest management by biological means in Latin America (Sinclair and Martha 2001).

Africa

In Africa, biological control has been used for a long time to control invasive alien plant species (IAPs) (Olckers 1999). Besides this, devastating effects of locusts and grasshoppers on African agriculture had also realized the requirement of biological control (Groote et al. 2001). Application of fungal-based products of *M. anisopliae* has proven to be effective in pest management (Lomer and Prior 1992; Bateman 1997; Hunter et al. 2001). Studies of using viruses for control of insect pests are also reported, but their long-term effective commercial use is not well documented (Cherry 2004). In Africa, despite broad interest in the use of BCA, their availability to growers has until recently been very restricted because of limited demand, technical and financial constraints, and in-country regulatory frameworks (Cherry and Gwynn 2007). According to estimation, the annual biocontrol sales for the whole of Africa in 2003 were approximately $23 million, including $5 million for bacterial products (Guillon 2003). In Kenya, for example, in 2002, the total pesticide sales were valued at approximately $57.4 million, of which $1.15 million (2 %) are accounted for by BCA sales, predominantly Bt-based products (Wabule et al. 2004). Overall, the data are not very well documented for Africa.

Global Market

Biopesticide companies have invested billions of dollars to develop a variety of microbial products so as to eradicate crop diseases. It is impossible to starkly tell the market trends for biopesticides, and there is a considerable discrepancy in both predication of global sales and selecting category of biopesticides. Yet some reliable data sources are market survey websites and biopesticide companies. The biopesticide market is growing at more than 20 % per year, and there may be a tremendous increase possible in the next 5 years (Market and

Table 2.2 Worldwide biopesticide sales

Biopesticide	Estimated sales figures (in $US million)					
	North America	Europe	Asia and Australia	Latin America	Africa and Middle East	Total
Total Bt (products based on *B. thuringiensis* serotypes)	72.0	27.57	74.75	30.19	6.28	201.79
Other bacteria	23.94	6.30	14.05	4.56	0.40	49.25
Viruses	5.57	7.47	23.90	3.80	0.48	41.22
Fungi	15.85	5.64	18.85	35.96	0.78	77.08
Nematodes and other	9.4	7.50	0.95	0.16	0.13	18.14
Total	126.76	54.48	132.5	74.67	8.07	396.48

Source: CPL Business Consultants (2010)

Market 2013). In comparison, there is a gradual decline in synthetic, and the overall market for the biopesticides had increased from $672 million in 2005 to over $1 billion in 2010, at an annual average growth rate (AAGR) of 9.9 % (Industrial Equipment News 2013). A more detailed report by BCC Research (2012) expected biopesticides market to total $2.1 billion in 2012 which may exceed beyond $ 3.7 billion in 2017, with a compound annual growth rate (CAGR) of 12 %. An estimation of global biopesticide sales by CPL Business Consultants (2010) is given in Table 2.2.

Biopesticide Resources

In the field of biocontrol, modern technology and digitalization of print media offered quick access to several resources dealing with the matter of interest. There are myriads of literature available free of cost in the World Wide Web (www). Although this e-service has been very popular in developed countries, various databases and online services have been developed with the motive to benefit farmers and researchers so as to provide ampule amount of information indiscriminately. A summary of the databases and services are mentioned in Box 2.1.

Constraints with Biopesticides and Possible Remedies

Since biopesticide is a product generally with live organism(s), utmost care is needed, at all the steps, beginning from the production till the end use to maintain the microbial load and vigor. Production technology of biopesticide requires proper care and aid of sophisticated equipments to ensure availability of quality products in the market. As discussed again and again, biopesticides although offer a great promise are still not able to perform up to the mark, and in fact it will not be wrong to mention that these eco-friendly products have not taken market by storm and are performing below par. Kabi (1997) gave stress on the production of quality biopesticides, since they are important in rendering sustainability to farming systems. Insufficient knowledge, lack of adequate machinery, inappropriate handling and improper distribution, importation laws for live inoculants, and several other issues can lead to lack of quality products and loss of market. Major constraints associated with biopesticide development and growth are discussed in this section.

Lack of Awareness

Agriculture market is witnessing an increase in demand for environment friendly, chemical residue-free organic products. Growth in some regions is however hindered due to well-established chemical pesticide markets, lack of awareness about benefits of biopesticides, and uneven efficiency of biopesticides. The lack of awareness, knowledge, and confidence in farmers is one of the chief reasons for the lagging of these eco-friendly pest control alternatives. There are lots of ifs that farmers observe while using biopesticides; the results are sometimes not homogenous or consistent, and hence the users find themselves

Box 2.1: Biopesticide Resources

International Biocontrol Manufacturers Association (IBMA)

IBAMA is the worldwide association of biocontrol-based industries producing microorganisms, macroorganisms, semiochemicals, and natural pesticides for plant protection and public health. IBMA has 180 members and participates in the activities of international organizations such as OECD, FAO, WHO, the International Forum for Chemical Safety, the European Commission, etc. The main objective of IBMA is to associate the international organizations and manufacturers that are involved in the development and use of biocontrol agents. IBMA also maintains the product quality of the biocontrol agents and also forms and maintains the ethical professional rules. IBMA transfers the information for biocontrol agents to the interested parties and also organizes training program (details periodically updated at website: http://www.ibma.ch/news.html) to improve the skill of the company staff members for the better research. IBMA has four divisions; they are microbial biocontrol agents, natural and biochemical products, semiochemicals, and invertebrate biological control agents. IBMA provides a platform for the biocontrol products companies to share the knowledge and accordingly improve their business performance.

Online Information Service for Non-Chemical Pest Management in the Tropics (OISAT) was launched by the Pesticide Action Network (PAN) Germany in 2003, with the aim of limiting the use of hazardous pesticides and providing safer alternatives to poor farmers (Carina Webber 2008). OISAT info is a web-based information tool offering trainers, extension workers, and farmers a quick access to up-to-date information in the form of illustrations, photographs, and glossary terms in order to minimize pest damage in a safe, effective, and ecologically sound way. Every report or articles before publication undergoes a peer review by experts working on related fields then can be publically accessed at www.oisat.org

The International Organization for Biological Control (IOBC) was established in 1955 as a global organization affiliated to the International Council of Scientific Unions (ICSU). This nonprofit organization is known for its leadership in environmentally safe methods of pest and disease control and establishing a quality standard in the field of classical biological control (Cock et al. 2009).

Biopesticide Industry Alliance (BPIA) founded in 2000 with the mission of increasing awareness of biopesticide and to deliver full range of benefits of biopesticides in pest management program. BPIA is also involved in facilitating global acceptance, successful development, and commercialization of biopesticides. BPIA is a standards committee that evaluates and recommends quality and efficacy standards for biopesticides in agriculture, forestry, turf and ornamental, public health, consumer, and other target markets, even industry.

The Bio-Pesticides Database (BPDB) was developed by the Agriculture and Environment Research Unit (AERU) based at the University of Hertfordshire, the UK, is a comprehensive relational database of basic identification, physicochemical, toxicological, ecotoxicological, and other related data for both the more traditional agricultural pesticides (PPDB) and veterinary substances (VSDB). This database provides around 450 records of biocontrol agents ranging from naturally derived substances to insect predators (website http://sitem.herts.ac.uk/aeru/bpdb/index.html). BPDB retrieved information at global level from various resources such as scientific literature and databases, manuals, registration databases dossiers, company technical datasheets, and research projects. Before uploading data on the website, its quality is assessed that involves cross checking and peer review by experts.

confused in adopting these greener alternatives. Microbial pest control products require more attention of farmers than chemical products. Condition is worst in developing countries where most of the farmers are even not familiar with the term "biopesticide" and lack efficient skills to practice and use them (Alam 2000).

Research and development of biological pest control methods must be given priority, and people in general and agriculturists in particular must be educated about the handling and use of such control measures. It is absolutely necessary to create awareness, among the end users (farmers), regarding the use, efficacy, benefits, and importance of biopesticides, and also harmful effects of chemical pesticides must be known and explained. The responsiveness could be achieved by introducing certain extension activities such as organizing teaching programs, workshops, and entrepreneurs dealing with the idea of promoting sustainable agriculture using biological products (Amin 2013). Guidance, explanation, and monitoring should be done regularly by proceeding for interactive questionnaire sessions that will inculcate knowledge and learning in the farmers regarding the use, application, and handling of biopesticides. Farmers should also be trained for the methods of application in fields, and some encouraging activities can be introduced such as providing rewards to the farmers who applied biopesticides or promoted their use (Halim and Ali 1998). The efforts of various government agencies to popularize the use of biopesticides will definitely have impact in elevating current status and application of biopesticides all over the world. The National Farmer Policy (2007) in India has strongly recommended the promotion of biopesticides for increasing agricultural production and sustaining the health of farmers and environment. It also includes the clause that biopesticides would be treated at par with chemical pesticides in terms of support and promotion.

The use of transgenic crops like Bt crops is also not up to the mark especially in developing countries. In transgenic crops, functional foreign gene is incorporated by biotechnological tools. Objections to development and deployment of transgenic crops by farmers rest on several issues related to ethnic matters, associated risks, lack of confidence, costs, market control, etc. Farmers generally lack faith in the use of transgenic crop. Hence, it is essential that training and teaching should be given to the farmers in regard to the use of transgenic crops. Special schools should be established particularly in the rural areas so as to train and teach the farmers to efficiently use the biopesticides. Proper application of biopesticides is very important to achieve the optimum results. Farmers need to be made aware of the implications of chemicals on the current and future prospects of soil and yields. These goals can be achieved by the support of government and corporate houses.

Lack of Faith and Inconsistent Field Performance

Lack of faith in the use of biopesticides was found to be one of the major factors responsible for their lagging behind (Arora et al. 2010). Many farmers who stopped using biopesticides reported that it was mainly because the supply was extremely unreliable and the performance very inconsistent (Alam 2000). A key factor involved in the lack of success has been the rapid decline in the size of populations of active cells, to levels ineffective to achieve the objective, following introduction into soil. Abiotic soil factors (e.g., textural type, pH, temperature, and moisture) exert their (direct) effect on inoculant population dynamics by imposing stresses of various natures on the living cells introduced in the fields (Evans et al. 1993). They can also act indirectly by affecting the activity of the indigenous soil microflora. Hence, maintenance of sufficient activity of inoculated populations over a prolonged period after release often represents the main hurdle in the successful use of microbes as biopesticides (Arora et al. 2010). Furthermore, efficient introduction into soil during the growing season is a major technical constraint. It is extremely important that a minimum effective threshold population of the introduced biopesticide is maintained in the soil/rhizosphere so as to combat the pests and pathogens (Arora et al. 2010).

Building farmer's faith and confidence by developing appropriate stress-tolerating formulation can increase product stability, constancy, and viability that can reduce inconsistency in natural field conditions. Production technology, employed for designing biopesticides, should be improvised along with sophisticated quality control measures and monitoring facilities. The designed biopesticide should be reliable, specific, indigenous, and replicable in its activity. Extensive research should be conducted in the fields to develop appropriate formulation working efficiently under diverse in vivo conditions (Retchelderfer 1984; Greaves 1993). Technical and chemical compatibility along with innovative application methods is a prerequisite for the success of a new biopesticide product in the agricultural industry. Developed formulation should be compatible with crop production practices and equipment. The host range and abiotic conditions under which the formulation is most effective must be clearly mentioned on the packets and if possible explained to the farmers.

Poor Quality and Shelf Life

Poor quality and performance are also one of the serious problems that hindered biopesticides takeover on the market. Several workers reported that the biopesticides being sold in the market are contaminated and have a low count of microorganisms (Singleton et al.1996; Alam 2000; Arora et al. 2010). Due to low bacterial count, it is not surprising that their performance is poor, deprived, and uneven. Due to which the shelf life is low and inconsistent in performance resulting in decline in the demand.

The inconsistent and seasonal nature of the existing demand requires efficient storage. The storage of biopesticides requires special facilities and skills, which most producers, shopkeepers, and farmers do not possess. Shelf life is a cessation (end) of several factors like production technology, carrier and packaging material used, and mode and distance of transport. All these levels are desired to sustain the shelf life. Bacterial survival in the desired formulation is affected by several variables: the culture medium used for bacterial cultivation, the physiological state of the bacteria when harvested from the medium, the use of protective materials, the type of drying technology used, the presence or absence of contaminants, and the rate of dehydration (Paul et al. 1993). Extreme care should be taken throughout the designing process to minimize the chances of contamination. It is also important that precautions should be taken to avoid adulteration during packaging, storage, and application of biopesticides.

Future research efforts in formulation technology should emphasize processes that will achieve viable and stable biological products. The most suggested solutions to the problem of survival time are air-dried and lyophilized preparations of biopesticides (Nakkeeran et al. 2005). Decrease in the water content in the biopesticides can be used for long-term survival during storage. In this way, the bacteria in the formulation remain inactive, resistant to environmental stresses, insensitive to contamination, and thus become more compatible with chemical pesticide applications (Bashan 1998). However, dehydration phase is also the most sensitive part of the entire formulation process, especially for nonspore-forming bacteria (Shah-Smith and Burns 1997).

Hence, it could be suggested that the main factors that have potential to affect economic feasibility of the biopesticide product are designing and optimizing perfect formulation technology. Good formulation can be reflected by the long product storability. Several commonly used biopesticide formulations with extended shelf life include granules, pellets, and dry powder based. Granules can protect the active agent from desiccation and also provide basic food for the agent. Powder is easy to apply by suspending it in water and also can cover a wide area of application (Urquhart and Punja 1997; Amin 2013).

High Budget of Production and Lesser Agribusiness

Hi-tech instrumentation required for producing biopesticides under completely sterile conditions is not getting acceptance. Screening of suitable

strains and research and development issues add to the budget. Large-scale screening of strains with biological activity is still required (comparable to more than 1:20,000 screened molecules for a new chemical product) (Bashan 1998). High sensitivity to temperature and other external conditions of these "living" inputs calls for enormous caution at the stage of manufacture/culture, transportation/distribution, and application. This involves investment in packaging, storage, and use of suitable carrier materials (Arora et al. 2001, 2010). In general, firms with larger production facilities are expected to invest more on networks to understand and access the market. All these factors prove that the raw material and instrumentation facility initially required for the biopesticide production are costly, and companies will only develop these products if there is a long-term profit in doing so. However, consistency and long-term returns can reduce the cost and enhance the profits.

A number of features of the agricultural economy make it difficult for companies to invest in developing new biopesticide products and, at the same time, make it hard for farmers to decide about adopting the new technology (Chandler et al. 2011). Most of the established companies relinquished their wish to do business in microbial pesticides but finally left the field due to huge losses in the agribusiness (CPL Business Consultants 2006). Commercial aspects of biopesticide industries were studied by various workers (Warrior 2000; Benuzzi 2004; Gelernter 2005) that confirm limited success and huge expenditure (Stewart 2001; Hallett 2005; CPL Business Consultants 2007; Droby et al. 2009). The market's potential in business decision of biopesticide industry is now being forecasted by leading global management consulting and market research firms that suggest that the agribusiness dealing with biopesticides requires huge intake of money, high-risk factors, and less profit (Leng et al. 2011).

Henceforth, the profit in biopesticide business could be made only by using novel techniques and tools. Agribusiness companies need to use certain innovative techniques and cheap raw material to capture or protect market shares by offering new products that buyers want (product innovations) and also by cutting costs (process innovations) and minimizing risk factors. Multifaceted bioformulations based on microbial consortia with diverse activities can be useful in bringing down the costs. Biopesticides designed from consortia will have multiple and holistic applicability in promoting plant growth; protecting plant health; strengthening plant-microbe associations under stress, pollutant, or contaminant-affected regions; and protecting plants from the attack of phytopathogens through biological control (Arora et al. 2013). Advanced countries take advantage of technology, using a variety of procedures such as licensing, buying, and accessing what others develop (Pray and Nagarajan 2010, 2012). Product protection by patenting and support by government in taxation and infrastructure development can also help.

Regulatory Framework

Registration of biopesticide is the main hurdle in the development, and most of the times registration is much more expensive than the production. Registration is not only expensive but also time-consuming (Ehlers 2006). The main problem is that biopesticides contain active cells (live organisms), and these live forms are treated like pathogens by the government agencies. Another issue is regarding the import and export of biopesticides; again, it should be pointed that export and import of chemical pesticide is much easier (as no one doubts on its integrity) in comparison to the use of biopesticides.

The assessment of risks is important because it provides the basis for governments' decisions whether to approve or register new biological pesticides and whether to renew the registration of old ones. Registration requires collation of data and preparation of dossier for submission to a national regulatory authority. By these efforts, governments can speed up the process of approving safer new pesticides and stopping use of riskier ones. Some major countries and their registration details are provided in Table 2.3. USEPA encourages development and use of

Table 2.3 Biopesticide regulation structures in different countries

Name of country/ continent	Registration/governing bodies
The USA	The USEPA is the main authority to regulate the use, sale, and distribution of conventional chemical and biological pesticides. USEPAs do this primarily from three statutes: (1) the Federal Insecticide, Fungicide, and Rodenticide Act (FIFRA) as amended, (2) the Federal Food, Drug, and Cosmetic Act (FFDCA), and (3) the Food Quality Protection Act (FQPA) of 1996. All ensure that pesticides use does not cause unreasonable adverse effects to humans or the environment
Europe	Here, the term "biopesticide" generally includes products with active substances based on microorganisms, botanicals (or biochemicals or plant extracts), and semiochemicals (including pheromones) and regulated under the Council Directive 91/414/EEC. This directive provides a list of active substances authorized for incorporation in plant protection products and lays down the requirements for application dossiers for new active substances and new plant protection products
China	In 1997, The Regulation on Pesticide Administration was introduced (revised in 2001 and 2004) to supervise and control manufacturing, marketing, and use of pesticides/biopesticide and agrochemicals in China or import to China
Australia	Registration of pesticides is governed by the Agricultural and Veterinary Chemicals Code Act 1994 and administered by the Australian Pesticides and Veterinary Medicines Authority (APVMA). The importation of a biological agent also requires authorization from the Australian Quarantine Inspection Service (AQIS) prior to introduction. If the organism has been genetically altered, approval from the Office of the Gene Technology Regulator (OGTR) is required prior to importation or release
India	Biopesticides fall under the Insecticide Act (1968). Central Insecticides Board (CIB) and the Registration Committee (RC) are two "high-powered" bodies under this Act. CIB is the Apex Advisory. It comprises eminent scientists of all disciplines/fields concerned. Whereas, the RC grants registrations to the persons desiring to import or manufacture insecticides, after scrutinizing their formulae and verifying claims with respect to their bio-efficacy and safety to human beings and animals
South Korea	Registration at first level is governed by the Agromaterials Management Division (AMD) and the Rural Development Administration (RDA), and then a new record is evaluated by Pesticide Safety Evaluation Division (PSED) and National Academy of Agricultural Science (NAAS). Once this dossier is deemed to contain sufficient information, the PSED holds two technical expert committees for product management and safety management. The committees provide the examination results to the PSED, who adjust the results and reports to the AMD and RDA. The AMD have a dedicated council for agrochemical safety for the final decision and for reporting to the applicant's dossier

Source: Kabaluk et al. (2010)

biopesticides. Since biopesticides tend to pose fewer risks than chemical pesticides, EPA generally requires much less data to register a biopesticide than to register a conventional pesticide (Kumar 2012).

The governments can frame regulations at the global level by organizing meetings, workshops, and conferences regarding uplifting the status of biopesticides/bioformulation. Governments should set up regulatory framework that could be accepted globally. Presently, different countries have different rules, and regulations due to which problems related to registration, use, import, and export do occur. The regulation can set up uniform acts or laws that could be accepted globally, so that there is a common policy regarding the use of biopesticides.

Health and Ecological Risks

There may be some chances of adverse health effects if biopesticides are not used according to the instructions mentioned on the product, but in comparison to chemical pesticides, risks are far lower. Biopesticides containing Bt as active

ingredient are not reported to show any major adverse effects on human health, but in some cases, occupational exposure confirmed health risks (Green et al. 1990; Bernstein et al. 1999; Doekes et al. 2004). Studies on fungal biopesticides suggest that spore of entomopathogenic fungi such as *Trichoderma*, *M. anisopliae*, and *B. bassiana* may cause allergy to farmworkers (Iida et al. 1994; Darbro and Thomas 2009). Recently, studies on mice confirmed as a robust fungal allergen from biopesticides of *M. anisopliae* (Ward et al. 2009, 2011). *M. anisopliae* is also reported to affect survival of nontarget pests (Thungrabeab et al. 2006).

Thus, it is necessary that before developing a biopesticide, strain monitoring should be extensively done. Doubtful strains should be screened out, as adding such microbes may result in the loss of confidence. However, care must be undertaken to ensure that any newly introduced natural product, being a microbial agent or secondary metabolite, should possess no threat to the operator, the environment, or the consumer before it is introduced into crop protection systems (Copping and Menn 2000). Apart from it, governments should also set up defined standards and permissible limits in regard to using biopesticides so that it diminishes the health risks. Essential data regarding the composition, toxicity, degradation, and other characteristics of the biopesticides is important and should be submitted to the respective agencies of various countries. In the USA, the registrants have to submit to EPA so as to ensure that the pesticide is safe for use. EPA conducts rigorous reviews to ensure that pesticides do not have adverse effects on human health or the environment (Kumar 2012).

Problems Associated with Viral Biopesticides

The main problem associated with viral biopesticides is the requirement of live host, tissue, or cell line culture for the proliferation and cultivation. Development of tissue culture laboratory in an institute (that works on viruses) again needs ethical clearances from the government organizations (Lapointe et al. 2012). The main cons of viral biopesticides are the culturing techniques, handling errors, and upsurge costs that ultimately hamper growth, production, and manufacture.

In all viral pathogens, including the most common biopesticides, the baculoviruses, replication is dependent upon the availability of permissive host cells. The accessibility and susceptibility of host cells to viral invasion and replication are classified into three categories: permissive, semi-permissive, and nonpermissive. In semi-permissive, infections result in limited viral progeny resulting from defects in some replication events, such as gene expression or viral DNA replication. In nonpermissive infections, cells do not support viral replication, and the process does not yield infectious progeny (Lapointe et al. 2012). Determining what factors influence the level of permissiveness of an insect cell to a particular baculovirus has proven to be challenging because baculovirus host range is affected not only by the interactions between the baculovirus and the host cell at the molecular level but also by aspects of insect behavior and physiology (Miller and Lu 1997; Cory and Hoover 2006; Thiem and Cheng 2009). Research and innovative techniques are required so as to ease the production, reduce the cost, and simplify the application of viral pesticides.

Competition with Chemical Pesticides

All over the world, chemical pesticides are used in very high amounts (Donaldson et al. 1995), and one-third of the agricultural production is dependent on pesticides (Liu et al. 2002). According to USEPA, over 1 billion tons of pesticides are used in the USA every year, and this is 22 % of the estimated 5.2 billion pounds of pesticides used worldwide (USEPA 2011). Consumption of pesticides in some of the developed countries is almost 3,000 g ha^{-1} (Khater 2012). Practically, biopesticides are not as effective as chemicals. Ahmad et al. (2007) compared the effectiveness of *B. thuringiensis* with, megamos (chemical pesticide) in controlling thrips

(*Thrips tabaci*) on garlic (*Allium sativum*) and found maximum yield using megamos treatment. In case of chemical pesticides, lesser quantity is suffice to kill a vast quantity of pests which is the main reason why farmers choose chemical pesticides over biopesticides. There have been several cases where chemical pesticides reduced losses of many crops, and in their absence it is reported that global losses would have risen from present levels of around 42 % to close to 70 % (Oerke et al. 1994). Knuston et al. (1990) provided further detail that in the absence of chemical control of weeds in wheat production, US yields would fall by 30 % and 5 % in the absence of fungicides and herbicides (Knutson et al. 1990). Similar studies also confirmed chemical dependence for the production of major crops around the globe (Farah 1994; Warren 1998; Webster et al. 1999; Aktar et al. 2009). Some workers are working on synergistic action of microbial biopesticides and chemical pesticides by IPM programs (Irigaray et al. 2003; Koppenhöfer and Fuzy 2003). IPM can also result in gradual decrease in the use of chemicals leading to development of confidence among farmers for the biopesticides. Research on combining microbial biopesticides with synthetic pesticides has showed improvement in control of some pest species including pesticide-resistant varieties (Khalique and Ahmed 2001; Cuthbertson et al. 2005). By removing the previously mentioned hurdles and constraints, confidence for biopesticides can be developed. This will only enhance the market for them. Quality products with the ability to act in field conditions will be able to compete with the chemicals and gradually overtake the market.

Conclusion

Since inception of biopesticides, their position and situation still remains in dilemma. Farmers find themselves confused and less confident in selecting biopesticides over the synthetics. Despite the fact that presently biopesticides are being used everywhere in the world, it is also known that developed countries seem to be ahead in their wider application (Chandler 2011). Developing countries have huge possibilities for using biopesticides as the production can be less expansive and labor is cheap in comparison to developed nations (Roettger and Reinhold 2003). Also countries like India are vastly dependent upon agriculture for not only feeding their populations but also for the economy which depends majorly on this sector. However, most of the challenges faced for the upliftment of biopesticides are fundamental and cosmopolitan. These include the efficacy of the microbial activity, survival of microorganisms, delivery systems, determining host range, and avoiding injury to nontarget organisms, consistency, performance in field conditions, economics, government regulations, and confidence among the end users. Gelernter (2007) has described the future of biocontrol in Asia, and according to him unreasonable expectations for performance, inappropriate regulatory guidelines, lack of documentation on the uptake of microbial control strategies, difficulties in implementing local production schemes, and inhibition of scientific exchange are the main hurdles in establishment of biocontrol. Biopesticide production also faces problem of quality control, and at the global level there is no uniformity in processes and methods. In this regard, Van Lantern (2003) starkly emphasized that the characteristics that affect overall quality have to be identified and must be quantifiable and relevant for the field performance of the parasitoid or predator. Though a lot of research is going on biopesticide development, but still it is further needed to be emphasized and explored (Gaind and Kaushik 2008). This exploration could be done by developing strong policy and encouragement from governments to the industry as well as end user by means of liberal tax benefits, incentives, etc. Creating awareness among the farmer community about the beneficial effects of BCAs and harmful effects of chemical protectants will certainly create a congenial and long-standing effect which can lead to commercial success of the biopesticides (Swati and Adholeya 2008).

In spite of all these limitations, biopesticides are gradually becoming popular, especially among local farmers, and that is why a statement of David Cary, executive director of the IBMA,

provides a hope of betterment that the biological control market "that is only 3 %, or $1.3 billion, of the $44 billion global crop protection business, is growing 10 % a year" (Patrick and Kaskey 2012). The growth of biopesticides is indicative of its importance in the sustainable agriculture by producing food crop with lesser chemical use. There is prediction that the world population will exceed nine billion by 2050, and efforts have to be made to meet the demand of 70–100 % more food from the same land area without the extensive use of chemicals (http://unsdsn.org/files/2013/05/130112-HLP-TG7-Solutions-for-sustainable-food production.pdf). Currently, the ultimate requirement for biopesticides to develop is to overcome the shortcomings which are associated with them. This reformation will certainly create similar acceptance as the synthetic pesticides and importantly without any adverse effect on the environment. A concerted effort of research institutes, universities, nongovernment organizations (NGO), and government organizations is required to elevate the stature of biopesticides. Determination at global level is required to strengthen these green alternatives and push off the red poisonous chemicals from our platter and of course the ecosystem as a whole.

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Biotrophic Plant-Microbe Interactions for Land Reclamation and Sustainable Agriculture Development

3

Vivek Kumar, Priyanku Teotia, Sandeep Bisht, and Shivesh Sharma

Abstract

Anthropogenic undesired actions intended at agricultural and technological advancement have led to the non-judicious creation and usages of various chemicals. Contamination of soil and formation of barren lands are a worldwide crisis, and reclamation of this using chemical or physical means is not a solution. The negative aspects of pollutants in the soil and environment lead to diverse impact on human beings, flora and fauna also. This undesirable facet relies on the pollution type, its severity and nature. The hunt for alternative methods for digging and incineration to clean contaminated sites resulted in the application of bioremediation techniques, but this is not cost-effective. The cost-effective and viable mode could be efficient utilization of plant-microbe interaction (PMI) pair in agricultural land reclamation. In the process of active rhizosphere functioning, root exudates of plant lead to proliferation, survival, and working of microorganisms, which subsequently results in a more efficient degradation of contaminants. The plant root system actually helps to spread microbes in the soil and assists in penetrating otherwise hard soil layers and surfaces. The inoculation of pollutant-degrading bacteria on plant seed can be an important additive to improve the efficacy of bioremediation or plant bioaugmentation. Biotrophic PMI is promising, a relatively novel technique employed in reclamation of the contaminated or degraded agricultural soils. It may be defined as the exploitation of efficient microbes along with their host plants to utilize or remove, obliterate, or

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impound hazardous chemicals at a particular site. This technology has so far been used experimentally to take away toxic heavy metals and other pollutants from contaminated soil; expansion of its capacity for applications to remove and degrade organic pollutants in the environment is the next phase. This chapter presents an overview of present aspects of microbes-plant relations in reclamation for feasible and viable augmentation of agriculture land biodiversity.

Introduction

Soil is an inadequate reserve, and its well-being is vital for any sustainable development in a particular area. Many developmental or unwanted anthropogenic activities have resulted in soil deprivation, and in the case of agricultural lands, there is successive loss of soil fertility. The characteristic of soil depends not only on its physical or chemical properties but also on the microbial diversity and activity of its flora and fauna (Doran and Linn 1994). From the last many years, enormous area of cultivable agricultural land has been degraded, neglected, and deserted. It has been estimated by the Food and Agricultural Organization that around 25–28 % of the world's land has developed some sort of dereliction, and this land is no more utilized for any sort of production. Countries in the Latin American region have developed heavily degraded land (around 27 %), all due to human activities (Bot et al. 2000). Among Latin American countries, only Brazil has around 236 million hectares of degraded land or approximately four times the area dedicated to arable crops. Degradation of these grazing lands, which were earlier rich and fertile agricultural areas, occurred chiefly owing to overgrazing, unattended vegetation burning, and non-application of chemical fertilizers in the soil. These soils were once fertile agricultural soil, which were uncovered in gullies, and did not have any organic matter to support microbial and plant growth, therefore resulted in poor soil health and physicochemical structure (Sharma et al. 2006). Comparable circumstances also happened in soils uncovered by road and rail corridors, land leveling on construction sites, and mining activities. Under these conditions, the revegetation of these areas by native vegetation is almost extremely limited. Moreover, the seeds from native forest species may be deposited by wind, water, or animal vectors, and many may germinate, they do not thrive, and spontaneous revegetation of the areas normally does not occur. In other situations these areas are invaded by persistent, fire-prone grasses that are known to preclude or severely slow subsequent forest regeneration (Parrota et al. 1997).

Reclamation of Land Polluted with Heavy Metals

Heavy metals are elements having some metallic characteristics such as tensile stress, conductivity, stability as anions, cations, ligand specificity, etc., and should have an atomic number more than 20. In any ecosystem, the most widespread and general heavy metals responsible for contamination are cadmium, copper, lead, mercury, nickel, arsenic, and chromium. Metals occur as natural components in soil, and many heavy metals are required by plants as micronutrients. As a result of human activities such as mining and smelting of metals, electroplating, gas exhaust, energy and fuel production, fertilizer, sewage and pesticide application, municipal waste generation, etc. (Raymond and Felix 2011), soil metal pollution has become one of the most severe environmental problems today. Heavy metal contamination of water and soils poses a major environmental and human health concern.

Efficient microbes and native plant interaction can be a gorgeous approach to attain the proficient taking away of pollutants in contaminated soils. Plants can be used to get rid of an ample

range of pollutants, because they can be monitored easily and many agriculture technologies are existing to lessen the costs of the treatment. The choice of micro-phytoremediation is usually cost-effective since it is innately and automatically driven (Rajkumar et al. 2012). Once this process is established, the contaminated sites typically involve slight financial input with regard to continuation. As heavy metals stay on place, the cost of transportation connected to shifting the soil contaminated with heavy metals to a treatment facility could be abolished. Scientists and agriculturist estimate that, on an average, the micro-phytoremediation technique is cost-effective compared to other available remediation methods (Pilon 2005). Abolition of transportation of heavy metal-contaminated soils also diminishes the chances of contaminants proliferating to other uncontaminated environments.

Micro-phytoremediation of heavy metals in contaminated soils may take one of several forms: micro-phytoextraction, micro-rhizofiltration, phytostabilization, and micro-phytovolatilization. Micro-phytoextraction is a method in which plants along with associated microbes help to uptake metals from soil into the roots and shoots of the plant. Micro-rhizofiltration is the employment of plant roots to uptake, concentrate, or precipitate metals from industrial effluents, and micro-phytostabilization is the use of microbes and plants to lessen the movement of heavy metals through absorption and precipitation by plant roots, therefore decreasing their bioavailability. On the other hand micro-phytovolatilization is the uptake and liberation of volatile compounds like mercury- or arsenic-containing components into the environment. The plant roots interrelate with a great number of diverse microbes, and these interactions play a major role in phytoremediation of heavy metals (Jing et al. 2007). The working efficacy of associated plant-microbe mutualism in a heavy-metal contaminated land can be influenced by associated microorganisms and type of plant. Microbes in soil play very important roles in plant nutrient recycling, upholding of soil structure, detoxification of harmful chemicals, biocontrolling of plant pests, and also regulation of plant growth (Kumar et al. 2000; Sandaa et al. 2001). Thus, the native potential bacteria can supplement the remediation aptitude of plants or diminish the phytotoxicity of the heavy metal-contaminated soil. Additionally, microbes and plants can form precise associations in which the plant provides the microbes with an explicit carbon basis that persuades the microbes to trim down the phytotoxicity of the contaminated soil. Plants and microbes can form nonspecific relations in which common plant activities encourage the associated microbial community, which results in the usual microbial metabolic activity, which biodegrades contaminants in the soil. Plants' root can supply exudates, which results in increasing ion solubility and then availability. These biochemical devices boost the remediation functions of microbes associated with roots (Marques-Ana et al. 2009). In a nutshell, the variation and adaptation abilities of plant and microbe as well as the bioremediation capability of the microbes are very crucial in reducing the damaging consequences of heavy metal pollution in any agricultural soil system.

Reclamation of Lands Polluted with Petroleum Hydrocarbons

Contamination of soil by petroleum hydrocarbons is an extensive, prevalent, and well-known worldwide environmental concern. While the majority of these hydrocarbon contaminants occur due to natural resource, release and accumulation of hydrocarbon contaminants through human act have led to noteworthy spoilage of agricultural soil as well as groundwater. A good number of common petroleum hydrocarbons in the environment are related with the handling and use of petrol, diesel, crude oil, and other oil products in automobiles and machineries employed in transportation and other industrial activities. The examples of hydrocarbons which are of environmental concern are gases (e.g., methane and propane, though these do not play a significant role in agricultural soil contamination or degradation), liquids (hexane and benzene, play a role as major contaminants), waxes (paraffin wax and naphthalene), and other polymers (synthetic

rubber, polyethylene, polypropylene, polyvinyl butyral, polyacrylonitrile, silicon, neoprene, and plastic or polystyrene). The three key classes of aromatic hydrocarbons of environment pollution concern are (i) polycyclic aromatic hydrocarbons, (ii) heterocyclic aromatic hydrocarbons, and (iii) alkyl polyaromatic hydrocarbons (McGuinness and Dowling 2009).

Although various forms of petroleum hydrocarbons are employed in modern industrial society, for exploration, extraction, purification, and transportation, use of petroleum products, however, poses inevitable environmental risks and menace (Pena-Castro et al. 2006). The presence of petroleum hydrocarbons (PHCs) in soils is not only an adverse factor for human health but also a negative impetus for plant growth (Meudec et al. 2007; Euliss et al. 2008). During microbe phytoremediation of organic contaminants, plants can further benefit from associated bacteria possessing appropriate degradation pathways and metabolic capabilities, leading to more efficient contaminant degradation and reduction of both phytotoxicity and evapotranspiration of volatile contaminants. Apart from environmental conditions the inoculation method and physicochemical properties of the soil are the main factors influencing the survival and activity of an inoculated strain as well as the growth of plants. Soil microorganisms act as biological catalysts for the bulk bioconversion processes and in few cases operate as exclusive and particular bioreactors to biodegrade the organic contaminants. An organic contaminant in the soil could be biodegraded by the process of oxidation or reduction, by the elimination of functional group of the molecule, or by the partial degradation of the crux structure of the hydrocarbon molecules. Mineralization is a method through which the natural and original organic molecules are degraded to generate carbon dioxide, microbial biomass, and inorganic compounds containing N, P, K, and S (Turco and Sadowsky 1995). Fruitful inoculation and use of selected potential soil microbes in feasible means is the initial action to improve the micro-phytoremediation of a contaminated soil. It is equally important that the microorganisms remain viable and can colonize roots and rhizosphere in order to continue improving plant growth and potentially degrade soil contaminants (Ramos et al. 2010).

Prakash et al. (2014) isolated, characterized, and evaluated the potential of PHC-degrading bacterial strains from oil-contaminated soil in Meerut region, India. Among 59 oil-degrading bacterial cultures isolated from oil-contaminated soil samples, one *Bacillus*, two species of *Pseudomonas*, and one species of *Micrococcus* were used for in vitro remediation of benzene, diesel, toluene, anthracene, and naphthalene. Maximum biodegradation of selected hydrocarbon, i.e., benzene and toluene, was done by consortium formulation. Moreover, *Bacillus* sp., APHP6; *Pseudomonas* sp., APHP9; *Pseudomonas* sp., APBP1; *Micrococcus* sp., APIO4; and their consortium resulted in 54.8 %, 60.2 %, 40.9 %, 32.5 %, and 66.2 % decrease in benzene concentration and 61.2 %, 68.4 %, 53.7 %, 39.3 %, and 75.4 % decrease in diesel concentration, respectively, after 6 days of incubation.

Bisht et al. (2010) isolated several naphthalene- and anthracene-degrading bacteria from the rhizosphere of *Populus deltoides*, which were growing in non-contaminated soil. Among these, four isolates, i.e., *Kurthia* sp., *Micrococcus varians*, *Deinococcus radiodurans*, and *Bacillus circulans*, utilized chrysene, benzene, toluene, and xylene, in addition to anthracene and naphthalene. In another study, Bisht et al. (2014) isolated eight endophytic bacteria from the roots of *Populus deltoides* growing in non-contaminated sites at the natural vegetation of Garhwal Himalayas, India. Among these eight, only *Bacillus* sp. SBER3 isolate was able to metabolize a wide range of polyaromatic hydrocarbons (PAH) and other hydrocarbons, i.e., anthracene, naphthalene, benzene, toluene, and xylene. Furthermore, quantitatively *Bacillus* sp. SBER3 was able to produce 22 mg ml^{-1} of IAA after 4 days and solubilized 0.96 mg ml^{-1} of available phosphorus, respectively, after 120 h; SBER3 also produced siderophore and 1 aminocyclopropane-1-carboxylate (ACC) deaminase. Along with these traits, SBER3 under in vitro condition inhibited the phytopathogenic fungi *Rhizoctonia solani*, *Macrophomina*

phaseolina, *Fusarium oxysporum*, and *Fusarium solani* with percent growth inhibition (PGI) of 60 %, 61.5 %, 64.3 %, and 12 %, respectively. Rhizoremediation potential of *Bacillus* sp. SBER3 was demonstrated in PAH-contaminated soil model system. Significant enhancement in shoot, root length, and root and shoot biomass including the stem girth of *P. deltoides* in comparison to control was also recorded, and concurrently endophytic isolate Bacillus sp. SBER3 degraded 45.6 % of PAH in the soil model system after 120 days. Such potentially PHC-biodegrading bacterial strains could be effectively employed in the rhizosphere of plants growing in contaminated soil for reclamation and reviving nearly biological dead soil. It is becoming increasingly clear that the mutualistic relationships that occur between plants and microbes especially in the rhizospheric zone could play a vital role in improving the efficiency of phytoremediation (Bisht et al. 2014). The addition of efficient microbes enhances the PHC elimination from soil, possibly by increasing plant germination and by stimulating the plants to grow easily and build up wider root biomass for more nutrients and water uptake.

Reclamation of Lands Degraded Due To Soil Erosion

A lot of many landmasses including fertile soil have suffered from soil erosion. In the broad background, the land not in its original undisturbed state is referred as eroded land. In the utilization milieu, eroded land means loss of biological agricultural productivity or erosion in the land's ability to hold enviable vegetation. Man-made fracas such as tunnels, roads, and building erections result in the diversified land erosion problems, soil fertility loss, slope creation, loose sedimentation and displacement of soil by air waterways, and finally disruption of vital indigenous habitat. The cost of development has to be paid in terms of land degradation, but, the gravity of the damaging impact may be diminished by the speedy reestablishment of aboriginal plants (Mackenzie and Mackenzie 1995).

To increase variety and diversity of plant species in any soil, the type of flora management or revegetation depends upon the status of soil disturbance. Microorganisms are highly distinct in the soil and offer an extraordinary challenge to scientists to understand their biodiversity. It becomes very challenging for researchers to assure a successful restoration of microbes, as compared to flora and fauna. Microbes should be given utmost priority of any restoration of biodiversity as they represent the vast majority of the minute but important diversity of an ecosystem at every site, but still the role of microbes is rarely considered in terms of maintaining an ecological diversity and stability (Blanco and Lal 2008). Native and potential microbes should be a center of attention, for restoring disturbed agriculture lands. Without most species of plants and animals, disturbed lands can become steady, and the majority of ecosystems would function normally, but without naturally occurring or introduced microorganisms, the natural ecosystem would stop functioning. For evaluation of microbial restoration and their diversity, there could be three critical points for measurement. The first point is to define the space and sequential allocation of different species and their affiliation to ecosystem progression. The second worry is to review the affluence of microorganisms within the different ecosystems. Finally, the third one is finding the ability for spreading and usual reestablishment for the requirement of man-made introduction of efficient microbes in the rhizosphere (Toy et al. 2002).

The role of symbiotic or associative microorganisms is much less varied and clearly plays decisive roles in the creation and perseverance of crop or forest plants including weed plant composition. Essentially there are two types of activities: one is plant pathogens that hinder plant growth, and the second one are symbionts that remove nutrient resources and exchange those resources with plants for energy, protection, and enriched plant root exudates. Many reforestation and replanting methods successfully established the native flora which reduced soil erosion. The purpose of these methods was to establish the native vegetation in eroded area with the help of

microbes and also not allowing the exotic plants to acclimatize in that area. The best tactic has been the planting of competitive and fast-growing native plants inoculated with effective microbes, and it could be bacteria or endophytic fungi. These symbiotic microorganisms augment plant growth by providing unavailable nutrients and other growth factors. The severe soil erosion results in consequent fine dust pollution. Fine dust directly or indirectly leads to chronic respiratory diseases (Tiittanen et al. 1999; Samet et al. 2000). This method has been successfully employed all over the world to combat land degradation by erosion (Rapp 1986). Since long, it has been presumed that degraded and deforested agricultural lands would relapse to their original vegetation in due course of time, and this practice commonly happens in temperate lands where reforestation occurs in the absence of domestic grazing and no man degradative activities (Weber 1986). In such soils natural microflora helps to rebuild the degraded ecosystem.

For reclamation of desert, plants, especially xerophytes, are exceptional top soil binders. They have the potential of stopping soil erosion and lessening dirt spread in deserted agricultural and town areas. Hence revegetation programs have to be correctly implemented to allow the growth of xerophytes in degraded areas. To cope up the demand of medicinal plants in the international market, it will be a better option to use degraded or eroded lands for their cultivation. This exercise will assist to rescue the lands from the overload of different unwanted activities besides being the economic exchequer for the growers (USDA 2014). However, the role of associated rhizosphere microbes should be monitored so as to utilize them along with the plants in reclamation activities.

Reclamation of Lands Polluted with Fly Ash

Combustion of coals in thermal power plants is one of the major sources of environmental pollution due to generation of huge amounts of ashes, which are disposed of in large ponds in the vicinity of the thermal power plants. This problem is of particular significance in India, which utilizes coals of very high ash content (approximately. 40–55 wt.%). Fly ash is a diverse complex combination of noncrystalline and crystalline solid phases and is generally a fine-powdered ferroaluminosilicate material with Al, Ca, Fe, Na, and Si as the predominant elements. Certain other metal elements like B, Mo, S, and Se are characteristically enriched in fly ash particles (Adriano et al. 1980). Presence of fly ash metals disturbs the natural characteristics of soil, which adversely affects microbial population and plant growth. High amount of fly ash causes hostile situation for flora and fauna to thrive in any ecosystem. Adjustment or correction of soil with some amount of fly ash is likely to influence the natural properties. Therefore, for land amendment purpose, if fly ash is to be employed, it is essential to seriously assess its possible consequences on the free-living as well as rhizospheric microbes, which play a pivotal role in sustaining the fertility and productivity of the agriculture land. Literature is very scant regarding impact of fly ash on biological activities of soil micro/macro flora and fauna. Limited and incongruous information is available on this subject. Garau et al. (1991) reported that application of fly ash to agricultural soil restrains the microbial respiration activity, nitrification, and mineralization rate. Pichtel and Hayes (1990) informed that microbial population and other biological activities are hindered by fly ash application.

Conversely, it has also been observed that the application of less amount of fly ash augments the biomass and variety of the soil microflora and enhances the aptitude of the agricultural soil to sustain plant growth and proliferation. Modifications in the soil characteristics using fly ash are also shown during microbial enzyme activity study, which increased up to 20–50 % in the presence of earthworms (*Drawida willsi Michaelsen*). It could be due to the fact that earthworm excreta acted as substrates for microbial growth and proliferation (Pati and Sahu 2004). Enrichment of vermicompost from nutrients points of view using N-fixing and P-solubilizing *Azotobacter chroococcum* has been studied and found that the enriched vermicompost could be

used to dilute the effects of unwanted factors of disturbed soil (Kumar and Singh 2001). Presence of simple organic compounds and N source is vital for proliferation of plentiful microbial population in fly ash-amended soils. Therefore, application of organic additives like press mud, sewage sludge, and bioorganic waste into fly ash-contaminated soil will certainly augment microorganism population by providing essential micro- and macronutrients along with a carbon source. Organic matter will mitigate the lethal effects of fly ash metals, balance the C:N ratio, and encourage the microbial population and their diversity (Pichtel and Heys 1990).

Fertility of various agricultural soils could be decreased at a shocking rate owing to the loss of organic substance as a result of poor soil biological health, soil erosion, and compaction and due to broad variety of metal pollutants present in fly ash. The physical, chemical, and biological characteristics of an agricultural ecosystem are influenced by the micro and macro flora. Presence of organic matter and sufficient number of active microbial population will recycle the nutrients and prevent the soil compaction and toxicity due to accumulation of chemicals by fly ash. However, in many cases adverse circumstances in troubled soils may lead to no or poor vegetation growth. Such disturbed soils can be restored using active rhizospheric microbial populations. Here plant growth-promoting microorganisms (PGPM) play a critical role by providing essential growth factors to the plants, overcoming abiotic and biotic effects and mitigating the toxic effects of contaminants in the soil. Disturbed soils with fly ash all over the world necessitate biological remediation approach (Schützendübel and Polle 2002). This approach is connected with a passionate hunt for aboriginal or exotic microbes, which can adapt in fly ash-amended environment and help the plant to grow, and directly or indirectly phytoremediation of the disturbed place will eventually be remediated. The arbuscular mycorrhizae (AM) fungi and plant growth-promoting bacteria can carry out this job efficiently in inconsiderate soil environment and encourage the bioremediation of bothered agricultural soils (Fomina et al. 2005; Baum et al. 2006).

The knowledge of efficacy and working aspects of soil inoculation of competent microbes is inadequate mainly because of lack of analyzing methods and also by unavailability of quick and efficient location-specific selection techniques. The greatest location-specific information will be generated by using active, efficient, and competitive free-living, associative, and endophytic microbes which are compatible with location-specific plants (Wenzel 2009). Well-suited potentially competent microbes can surely and significantly be a part of bioremediation of inorganic contaminants. This is the reason that screening of efficient microbial inoculums is a key foundation of triumphant micro-biotechnological application in the soil. Nevertheless, a number of impediments should be conquered to attain the unbeaten commercialization of microbial technology. The most pertinent and important study of soil microbes for consequent applications in bioremediation of fly ash-contaminated soils is the research trials and observations done in natural environmental conditions. In spite of this, only thorough genetic and molecular exploration of an individual competent microbe in highly accurate and proscribed environmental circumstances can explicate the fundamental and vital means of their influence on plants in fly ash soils. For this reason, all aspects of procedural ladder are significant for uncovering, elucidation, and following controlled exploitation of communications among plant roots and their associated soil microbes (Read 2002).

Role of PMI in Sustainable Agriculture

In any good soil, a distinctive equilibrium of chemical, physical, and biological aspects maintains perfect soil characteristic. A healthy agri-environment structure and performance is dominated mainly by soil microbial dynamic system. A balanced and vigorous soil microbial community that decomposes organic matter and recycles macro- and micronutrients results in a sustainable and productive agriculture ecosystem (Solanki et al. 2011). Therefore, effective

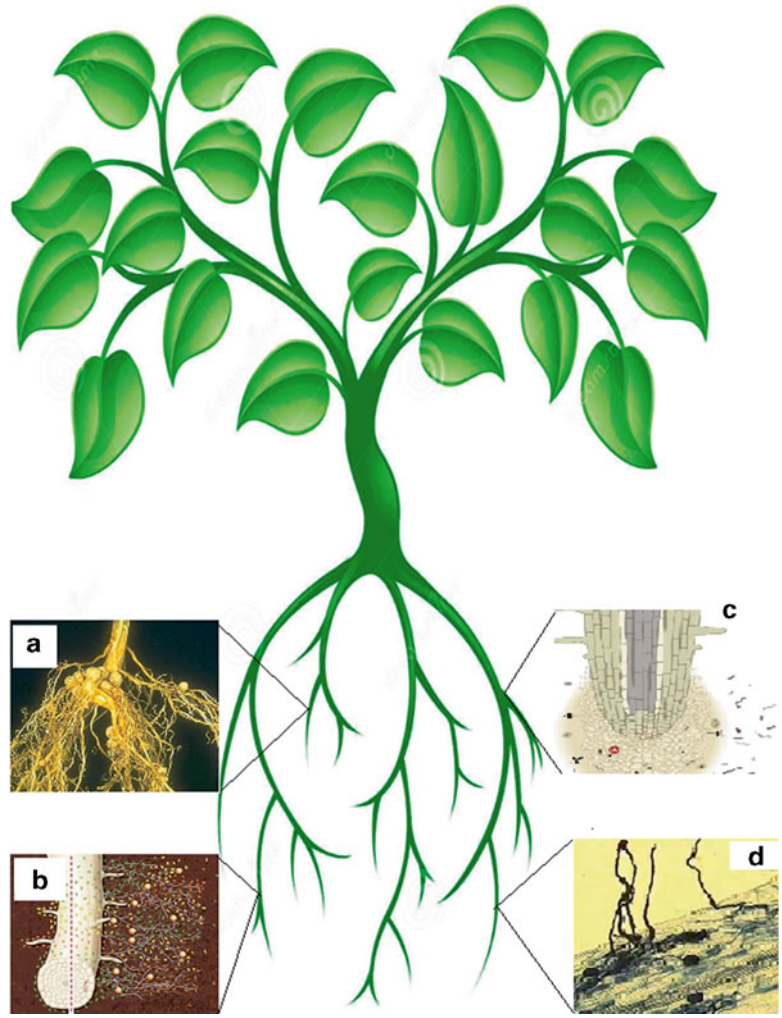

Fig. 3.1 Advantageous microorganisms in the soil, rhizosphere, and roots: (**a**) root nodule bacteria, (**b**) free-living and rhizospheric bacteria, (**c**) root exudates attract useful bacteria, and (**d**) AM fungi exploring soil for nutrient uptake

communications between the plants and saprophytic microbes are an important aspect to figure out the degradation of the eco-environment system and have key impact on the working and development of agricultural soil structure (Kumar et al. 2000). Modification in land use or types of cropping system can alter the microbial population constitution of the rhizospheric soil. Knowledge of influence of dissimilar crop plants growing individually or as a mixed cropping system, which could play a vital role in decisive composition or type of microbial population, able to effectively recycle the available nutrients and production of phytohormones and repression of plant disease is essential. An optimal balance and management of soil fertility is required for enhancement of agricultural sustainability and productivity (Johansson et al. 2004). The role of naturally occurring and competitive advantageous free-living, associative, endophytic microbes and arbuscular mycorrhizal fungi (AMF) needs to be fully exploited in agriculture, regardless of its sizeable stimulus on crop production (Fig. 3.1).

Many workers have reported that beneficial microbes have manifold functions in soil

ecosystem (Johansson et al. 2004; Kumar et al. 2013). These microbial communities are perfect means in soil for sustainable agriculture and restoration of degraded lands, forestry, and horticulture. On the other hand still there are research gaps as well in our understanding of microbial functions especially in the rhizosphere. Even without understanding and concentrating on managing the role of microorganisms, the growth of agriculture took place in the last several decades. It was not known that rhizospheric microbial community and their actions are indispensable for soil biological vigor and plant growth. Free-living as well as rhizospheric microbes are accountable for fundamental functions such as recycling of nutrients, N_2 fixation, P solubilization, biocontrol activities, and finally decomposition of organic, dead, and decay materials (Kumar et al. 2000, 2001).

The supervision of soil microorganisms in soil, through screening and inoculation of potential microbial species or by simply encouraging naturally accessible microbes, embraces immense assurance for sustainable and balanced agriculture system. For this, there are many reasons; firstly, microorganisms are versatile in functioning, ranging from biocontrol agents to plant growth promoter. Secondly, once introduced, microbes can perpetuate and survive well, with availability of favorable conditions. Finally, managing the microbial population system may encourage soil flexible systems. Existing agricultural ecosystems are insolvent in terms of their biodiversity and physiological and functional variety (Kumar et al. 2009, 2011). For maximum profitable utilization of microbial strains, cautiously controlled field experiments of crop plants are essential. An augmented understanding of plant-microbe association could impart the way for developing sustainable agriculture in order to guarantee food and feed production with nominal threat to the environmental system. To accomplish food, feed security, and sustainable agriculture production system, abovementioned aspects need promotion and awareness among common man and farmers.

Role in Soil Health

The health of soil ecosystem depends upon the diverse microbial community present, which contributes by transforming unavailable atmospheric N_2 into available form, solubilizes P, and provides other nutrients to plant for its optimum growth. Microbes also contribute a central role in nutrient recycling within the soil. Any uncontaminated soil contains plentiful genera of fungi, protozoa, and bacteria, which play an active part as biocontrol agents, phagocytizing pathogenic microbes. Using manifold means, plant growth-promoting microbes (PGPM) promote the development and maturity of crop plants exactly and implicitly. PGPM produce secondary metabolites, i.e., plant growth-promoting substances, which result in modifications of root morphology consequential in larger root exterior zone for maximum absorption and transport of nutrients to plants. Excretion of siderophores, by soil microbes, causes chelation of iron leading to competition for iron by soilborne plant pathogens. The larger root surface region for uptake of macro- and micronutrients and production of secondary metabolites may facilitate to optimize nutrient recycling during abiotic and biotic stresses due to unsuitable weather or soil conditions or due to competition with pathogenic microbes (Yang and Crowley 2000).

Many bioinoculums for leguminous and nonleguminous crops have been developed, which have resulted in awareness all over the globe. Commonly used PGPM bioinoculants of *Rhizobium*, *Azospirillum*, *Azotobacter*, *Bacillus*, *Pseudomonas*, AM fungi, etc., are commercially available for many cultivated crops. The purpose of PGPM is to recycle the available and unavailable nutrients in soil with minimum application of chemicals. Application of PGPM under field as well as in greenhouse conditions has encouraging responses; however, under field conditions, due to some unspecified reasons, applied bioinoculants do not perform as per expectations. For healthy soil system and development of competent microbes, the inoculation of PGPM is advisable in every cropping season after thorough research of soil and climatic conditions (Carvalhais et al. 2011).

Aboriginal plant species and their associative microbes due to their adaptation and acclimatization are doing well in environmental treatment as well as in healthy soil formation. Introduction of exotic species in the Thar Desert, India, and Sahel regions, Africa, for restoration of ecosystem and soil health did not generate encouraging results as exotic species could not fit into the new environment (Catterson et al. 1987; Sinha 1997) as compared to the aboriginal species. For rehabilitation of acidic or saline-contaminated soils especially in desert regions, importance should be given to those plants, which can survive or tolerate these harsh conditions and finally help in soil building and its productivity. Isolation and regular reinoculation of potentially competent microbes from such regions will certainly help to restore the soil health for many years to come.

Role in Abiotic and Biotic Stress

A variety of abiotic factors such as climate change, drought stress, pollution, and heavy metal contamination can influence the development of microbial relationships in the rhizosphere. In almost most of the cases, presence of mycorrhizae can alleviate the stresses to plants caused by these external influences. Inefficient mining processes, treatment of soil with sewage sludge or industrial effluents, and overuse of heavy metal-containing fertilizers or gas exhausts have, among other factors, contributed to the creation of large areas contaminated by heavy metals, radionuclides, and persistent organic pollutants. Such areas may jeopardize human health and ecology (Mathur et al. 2007) and, therefore, should not only be under strict control but should also be managed in a way which attenuates the possibility of any risk concerning the contamination of the food chain and the groundwater or air by wind erosion.

Soil Salinity Reclamation

In arid and semiarid regions, soil salinity is considered the most significant constituent of land degradation. It is an active process, spreading globally in more than 100 countries and covering on an average more than one billion hectares. Excess soil salinity leads to noteworthy degradation in fertile and irrigated agriculture lands due to poor understanding and mismanagement of natural resources. Recently, some noteworthy technological developments are available to evaluate soil salinity by using remote sensing technologies. The geographical information system, geophysical methods, and modeling, from regional, national to farm levels, also provide a major overlook to monitor land degradation due to salinity. The diverse types of soil salinity which influence agricultural productivity, like irrigation-induced salinity and "transient" dryland salinity, have been observed in desert regions of the world (Munns and Tester 2008). Obviously, soil salinity is one of the key ecological stresses that lead to reduction in agricultural productivity all over the world. Land degradation due to soil salinity and population growth has led agricultural scientists to develop salt-tolerant or resistant crops using genetic techniques (Yamaguchi and Blumwald 2005). On the other hand, the biochemical, molecular, and physiological means of salt resistance or tolerance in agricultural crops are yet to be understood in detail, and this is the reason that advancement in developing salt-tolerant crops has been sluggish. In the saline agricultural environments, soil salinity is often accompanied by additional abiotic and biotic stresses. For example, metals like high boron concentrations often happen in saline environments. Therefore, one has to understand the interactions between soil salinity and high boron toxicity in crops (Jahromi et al. 2008).

Plants are affected by salinity in various manners such as heavy metal or precise ion toxicity, physiological disorders, and also osmotic effects (Esechie et al. 2002). The optional approach to develop salt tolerance in plants could be by introducing salt-tolerant beneficial microbes that may augment crop production or help plants to thrive in adverse environment. Important role has been played by symbiotic relationships such as AM fungi, whose hyphal networks increase the soil surface area for the plant cells. The ectomycorrhizal fungi, which form a network layer around the root hair system and also in to the root intercellular

spaces, and rhizospheric plant growth-promoting rhizobacteria are known to play a very significant role under saline conditions (Manchanda and Garg 2008; Ben Salah et al. 2009). One of the main storage carbohydrates in the extraradical mycelium as well as in spores of AM fungi is trehalose. The biosynthesis of trehalose is reliant upon colonization of AM fungi with plant roots (Hoekstra et al. 1992; Schubert et al. 1992). Accordingly, the accumulation of trehalose might help to defend plants against salt stress. Romero et al. (2006) reported that silicon improves salt tolerance in plants by limiting Na^+ uptake through an unknown means, thus, improving photochemical efficiency and therefore, protecting cell membrane integrity, increasing antioxidant enzyme activity, and reducing water loss. Fascinatingly, the AM fungi *Glomus intraradices* is able to build up silicon and can improve its concentration in the host plant (Hammer et al. 2011), but the exact role of silicon in preventing water loss is yet to be deciphered.

The regulation of phytohormone production by microbes has been known in controlling plant vegetative growth during the osmotic stress by regulating the plant growth and plant leaf senescence (Kumar et al. 2001; Ghanem et al. 2011). Aroca et al. (2008) reported that AM fungi infection increases leaf abscisic acid (ABA) accumulation in drying soil, but limited information is available on whether AM fungi modify the ABA relations of plants growing in saline soil. The effect of AM fungi on plant ABA level seems contradictory, but ABA level in plants appears important in determining mycorrhizal fungi colonizing the plant root system. In one experiment, ABA-deficient tomato mutant, which accumulates at least 50 % lesser ABA concentration in roots compared to its wild type (Cornish and Zeevaart 1988), exhibited less root colonization by AM fungi *Glomus intraradices* DAOM197198 (Herrera et al. 2007). Some AM fungal species such as *Scutellospora calospora* attain maximum spore germination under high salt (300 mM NaCl) concentrations (Juniper and Abbott 2006; Jahromi et al. 2008). Application of potential microbes to enhance plant growth and yield in saline soil conditions entails that the inoculants should also be salt tolerant or resistant, which highlights the prospective of using microbes from saline habitats. Certain plant growth-promoting microbes, which can survive, proliferate, and colonize the root system in saline environment (Paul and Nair 2008), certainly offer substantial promise as bioinoculants.

It is a long-term objective to reclaim saline soil using improved and efficient plant-microbe pair for salinity-affected land. Plant survival can be accomplished with an understanding of the means of osmoadaptation in microbes and plants. Accumulation of amino acids and carbohydrates in microbes such as proline, glycine betaine, glutamate, and trehalose in response to soil salinity or high osmolarity results in better adaptation. Here proline plays a very prominent role in osmoregulation through change or adaptation in osmotic stress that shifts the dominant osmolyte from glutamate to proline in *Azospirillum brasilense* (Tripathi et al. 2002). Upadhyay et al. (2012) isolated ten species of *Bacillus* from wheat rhizosphere of saline soil. Proline content increased with NaCl stress, and maximum production was recorded with isolate SU8- 2.73 and 11.95 g mg protein at 0 % and 10 % NaCl (w/v), respectively.

Species of *Bacillus* have evolved highly sophisticated regulatory networks for protection against sudden unfavorable environmental changes, including nutrient malnourishment, changes in conditions such as temperature and humidity, oxidative stress, and abrupt increase in soil or surrounding salinity. Spore-forming bacteria such as species of *Bacillus* are one of the major types of soil bacteria that help in survival and proliferation of plants in saline environment mycorrhizal fungi being the other ones.

Drought Resistance

Due to excess and uncontrolled irrigation by farmers, water table has gone down, which has resulted in arid or semiarid conditions. In such conditions the role of microbes becomes very important to help the plant overcome the drought. The transpiration and conductance rate reduces in plant leaf due to water scarcity, the water use effectiveness increases, and using this technique

the plants survive over the drought period (Aroca and Ruiz 2009). An increase in ABA in plant leaf, which reduces water loss by stomata closing and leading to production of activated oxygen species, results in prevention of water loss (Cho et al. 2008). Additional plant-signaling components, for instance, salicylic acid, methyl jasmonate, and ethylene, also play a vital role in stomata closure and reduce the rate of transpiration. Techniques that permit plants to handle drought conditions are synchronized by gene expression alteration. Genes which manage drought conditions are of two types: (a) functional genes, which encode for transporter proteins and detoxify enzymes and biosynthesis of osmolyte enzymes, and (b) regulatory genes, which encode for transcriptional factors (Aroca and Ruiz 2009). In general, the favorable impacts of rhizospheric PGPM on plant water stress conditions are caused by alteration in phytohormonal contents, specially ABA, ethylene, and cytokinins. Inoculation of *Arabidopsis* with *A. brasilense* Sp245 exhibited higher ABA amount compared to uninoculated plants (Cohen et al. 2008). Arzanesh et al. (2011) used different strains of *A. lipoferum* (under drought conditions) to inoculate wheat seedlings. Inoculation with bacteria having properties of phytohormone production and phosphate solubilization alleviated wheat plant drought stress and improved plant development and growth yield (Kumar and Narula 1999; Narula et al. 2000). Bacterial production of hormone-like substances and their ability to stimulate plant endogenous hormone levels were believed to play a key role in this process (Dobbelaere et al. 1999). Under aerobic conditions, it has been observed that *A. brasilense* generates noteworthy amounts of diffusible gas, nitric oxide. These biomolecules have been reported to work as signaling molecules in an indole acetic acid-stimulated pathway, which is implicated in adventitious root growth (Creus et al. 2005; Molina et al. 2008). Bacterium, *P. polymyxa*, isolated from drought-prone *Arabidopsis thaliana* induced drought-responsive genes at a transcriptional point, which shows a role in overcoming drought stress. Plant inoculated with bacterium was further resistant to water stress compared to uninoculated (Timmusk and Wagner 1999).

PMI and Nutrient Uptake

The availability of nutritional material present in soil greatly influences the plant growth and yield in an ecosystem. Normally, a plant could face noteworthy remonstrance in harvesting a sufficient amount of these nutrients to suffice the requirement of fundamental and essential metabolic course owing to their unavailability and fixation in soil (Liu et al. 2011). A scarcity of a few one of them might consequence in poor or less plant growth. Indications of nutrient shortage could result in diminutive growth, plant tissue death, and small size and yellowing of leaves due to decreased amount of chlorophyll production, an essential chrome material for photosynthesis. The deficiency of nutrient supply to the plants will have adverse effects on agriculture, resulting in fewer yields with poor quality. It can also affect the overall eco-environment of a region including the soil microflora, whose survival and growth greatly depend upon the root exudates. There are chiefly two types of plant nutrients: macro- and micronutrients or trace elements. The macronutrients are the construction lump of vital cellular machinery, and parts such as nucleic acids, proteins, and carbohydrates therefore are needed in huge amount by the plant cells. Nitrogen, phosphorous, potassium, sulfur, and magnesium are crucial and vital macro elements for a plant. Micronutrients, such as Zn, Fe, Mn, Cu, Co, and Ni, are requisite in very little amounts, because these play an important role in enzyme functions and activity. Plant adopts different strategies for the uptake of Fe from soil, as depicted in Figs. 3.2 and 3.3.

Two major nutrients, N and P, are considered most important for the plant growth, yield, and development since they are frequently present in minute quantities or are available in a form that cannot be taken up by the plant (Vessey 2003). Consequently, the development and progression of most plant species resulted in the improvement of reciprocally symbiotic, helpful, and advantageous

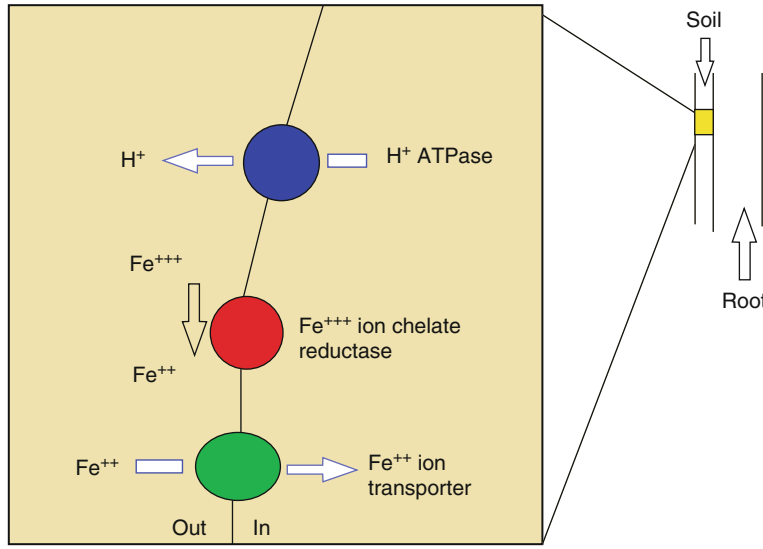

Fig. 3.2 Plants induce the activity of a proton ATPase, a ferric chelate reductase, and a ferrous iron transporter during iron limitation (without bacteria)

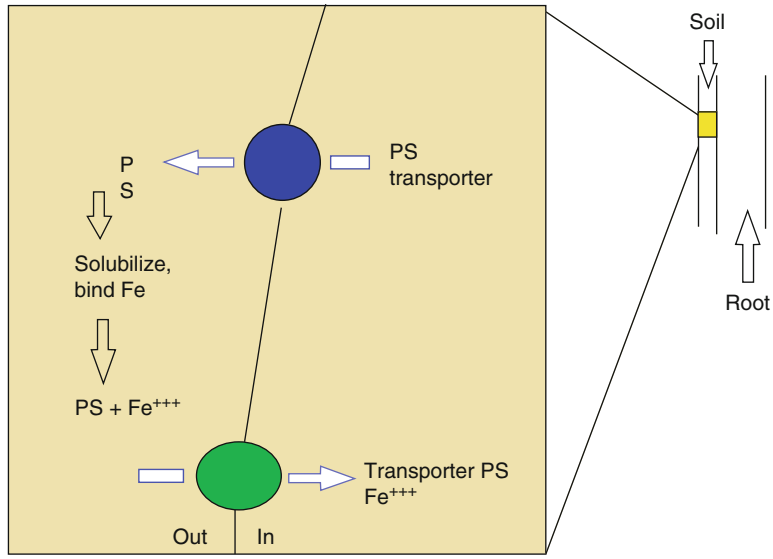

Fig. 3.3 Plants in association with microbes synthesize and secrete phytosiderophores (PS) into the soil during iron deficiency; the PS bind ferric iron complexes that are transported to plant root

relationships with soil microbes. The result of this relationship developed the dependency of plants and microbes on each other. Many associative, free-living, or endophytic microbes fix atmospheric N for the plants and also solubilize the fixed P making it available to the plant (Kumar and Narula 1999; Kumar et al. 2009, 2011, 2013). Diverse bacterial and fungal species have the potential to solubilize diverse insoluble inorganic P forms, such as tricalcium phosphate, dicalcium phosphate, hydroxyapatite, and rock phosphate. Such microorganisms are present in most agricultural

lands and constitute about 40 % of the culturable population (Richardson 2001). Among the common bacterial genera having this property are *Pseudomonas*, *Bacillus*, *Rhizobium*, *Burkholderia*, *Achromobacter*, *Agrobacterium*, *Micrococcus*, *Aerobacter*, *Flavobacterium*, and *Erwinia* (Rodríguez and Fraga 1999).

The limitations of diverse nutrients in soil also affect the rhizospheric microbial populations; this effect is due to the modification of root micro-rhizosphere environment (Chen et al. 2006). These differences are thought to relate to modifications in rhizodeposition patterns. A wide range of deficiency symptoms are observed in a plant in nutrient-limiting conditions. These nutrient limitations also influence the secretion of root exudates (Kumar and Narula 1999). The symbiotic microbes can noticeably play a vital function in approaching complicated organic nitrogen, nullifying the role of saprophytes in soil nutrient mineralization. In spite of this, in a number of mycorrhizal organizations, these saprotrophs cooperate with plants by providing them nitrogen. The mycorrhizal fungi increase N, P, and other nutrients in plants by increasing the surface area using hyphal extensions (Jonsson et al. 2001; Lerat et al. 2003). This augment in N, P, and other nutrient uptake is related to the encouragement of bacteria proliferating in the micro-rhizosphere of the plant.

Future Aspects

Novel technologies and approaches should be implemented that will aid to improve the biodegradation efficacy of microbes in the rhizosphere. Consequently, additional studies of the selection of potent rhizospheric microorganisms or communities, which can survive and proliferate in the root zone of a plant which is suitable for rhizoremediation or phytoremediation, could result in better and extended approach. This plant microbe scheme then can be an interesting means to develop and expand bioremediation into a widely accepted land area. The inoculation of genetically engineered microorganisms with unique pathways will create novel or improved functions, which can seize a great prospective and promise for increased phyto-/bioremediation. Bioremediation can also be improved by the employment of the genes responsible for the biosynthetic pathways that can augment biodegradation processes by enhancing the bioavailability of the substrates. Moreover, genes responsible for encoding resistance to important stress factors present in the ecosystem may increase both the survival and the proliferation of specially created microbes. As a result, genetic engineering of aboriginal microbes, suitably adapted to native environmental conditions, could make the bioremediation methods more feasible and eco-friendly. Importance should be given while developing bioremediation systems using a plant-microbe pair to select a native type of microorganism, which can enhance the process using natural gene transfer mechanism. This is also useful to evade the complications of national as well as international legislation matters regarding restricting, using, and monitoring the application of genetically modified microbes in a particular ecosystem. Nevertheless, with a universal political budget towards green and sustainable bioremediation methods, the employment of plant-associated microbes to biodegrade toxic man-made compounds or overcome natural inhibiting factors in degraded land may provide competent, cost-effective, and sustainable green technology. Another exhilarating choice could be to exploit the potential of plant-associated microbial genome for degraded ecosystem or biodegradable contaminated compounds, using the application of rDNA technology to create transgenic plants using bacterial genome. These plants can express bacterial genes resulting in enhanced plant lenience and metabolism of noxious organic compounds in a particular area. Some transgenic plants have been developed for phyto/bioremediation of petroleum hydrocarbons and heavy metals. Genetically engineered poplar plantlets excreting bacterial mercuric reductase enzyme have been shown to germinate and successfully grow in mercury-contaminated soil. Based on this line several other native plants can be modified using microbial genomes to grow well in contaminated or degraded soils.

Conclusion

Biotrophic plant-microbe interaction process is a hopeful approach to reinstate degraded lands. However, there are certain challenging issues which still linger on to be conquered. Certain amount of understanding is required to recognize how plants and microbes will react in the non-favorable or unfertile environment. There is still a lot of work to be carried out, more in open fields, especially in degraded ecosystems based on the laboratory and greenhouse studies. One has to provide commercially feasible systems for using plant-associative free-living, endophytic, and rhizospheric microbes to biodegrade a wide range of contaminated compounds and overcome abiotic and biotic factors in any environmental system. Beyond doubt, since long the soils have been built by plants and their associated microbes, the association of both of these has contributed in improving the physicochemical characteristics of soils to permit better development of plants and their associated microbes. This association has led to increased plant growth, depth and loosening of soil, and structuring; better water holding capacity; better aeration and gaseous exchange; increased availability of inorganic and organic compounds to roots; and enhanced rock weathering and nutrient recycling. There are also other key questions to work for novel crops and better cropping practices for efficient and better harvesting of nutrients from soil, especially P, which gets fixed in soil. The application of P-solubilizing microbes solves this problem to a great extent. The complex ecology of the rhizosphere is a potentially interesting research discipline, having numerous exhilarating defiance and remonstrance, equally with a basic and a strategically applied perception. Recognition of dissimilar techniques related to rhizospheric plant-microbes communication opened novel potential and chances to devise a tactic for increased crop growth and yields. Many researchers explain numerous advantageous effects of PGPM on plant yield and growth by changing the microenvironment of root rhizospheric system including nutrient supply and controlling pathogens. After this the use of micro-biotechnology techniques can be employed to make better PGPM strains by combining many beneficial features in one organism. Finally, one could question that modification of micro-rhizosphere by inoculation of competent PGPM will influence the natural microbes. Whether it will adversely affect the natural population or have synergistic effect is not clear at present. One has to increase the knowledge and information of such microbial interactions and their behavior before we exactly plan to introduce the competent or all in one PGPM in the rhizosphere for sustainable growth of the plant.

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Plant-Microbe Partnerships for Enhanced Biodegradation of Polychlorinated Biphenyls

4

Prameela Jha and Prabhat Nath Jha

Abstract

Our modern civilization rests on synthetic organic compounds. Some of these compounds which have become indispensable for human society are termed as persistent organic pollutants (POPs). They enter into the food chain where they bioaccumulate in fat tissues of organisms at various trophic levels and usually biomagnify the physiological effects at higher levels resulting in severe health hazards. Civil and mechanical engineering remediation methods deployed to clean such environmental contaminants have serious shortcomings of being expensive and environmentally invasive. Thus, there is an urgent need to search for alternative strategy(ies) which does not suffer from abovementioned shortcomings. The past decade has witnessed a momentum in the use of plants and its associated microflora partnerships as an alternative option. Plant and associated bacteria interact synergistically to lower down the levels of contaminants present in the immediate vicinity of root systems. Plants modulate root secretions and exudations to orchestrate recruitment of catabolically active bacteria in their rhizosphere. This recruitment in turn biodegrades contaminant(s) present in the rhizospheric soil by bacterial communities which subsequently reduces environmental stress on the plant. Thus, it is highly recommended to explore novel plant microbe pairs naturally operating in organic contaminated ecosystems and study the members of microbial communities to get more insight into a phenomenon of phytoremediation so that the technology may be optimized and extended from lab to land. The present review highlights the synergistic effects of plant-microbe interactions in the phytoremediation of organic pollutants with special reference to polychlorinated biphenyls (PCBs). Also, the state-of-the-art techniques used to study microbial diversity within PCB contaminated environments are discussed.

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Introduction

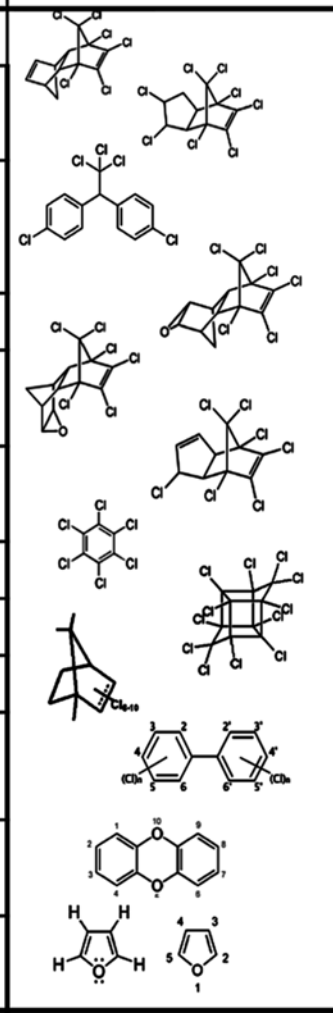
Comfort and convenience are the words that best describe modern-day human society. But to achieve comfort and convenience in every walk of life, we are synthesizing certain organic compounds which are not suitable for both humans and the environment. A wide variety of toxic chemicals including heavy metals and POPs are being indiscriminately dispensed into the environment. POPs are chemical substances that persist in the environment, bioaccumulate through the food web, and pose a risk of causing adverse effects to human health and the environment. These compounds have the capacity to travel long ranges from the source of origin and reach the places where they were never used or synthesized, thus, making them more dangerous. The Stockholm Convention on POPs, under the auspices of the United Nations Environment Programme (UNEP), an international agreement enforced in 2004 (Stockholm convention 2004), enlisted 12 organic compounds as POPS of major concern. These 12 POPs include PCBs, 9 chlorinated organic pesticides (aldrin, chlordane, dichlorodiphenyltrichloroethane (DDT), dieldrin, endrin, mirex, heptachlor, hexachlorobenzene, and toxaphene), and dioxins and furans. Other organic pollutants of major concern are industrial solvents, total petroleum hydrocarbons (TPH), polyaromatic hydrocarbons (PAHs), halogenated hydrocarbons, volatile organic compounds (VOCs), and brominated flame retardants (Table 4.1). The health hazards of these organic pollutants include skin irritation, endocrine disruption, increased risks of developing cancer and developmental disabilities, and increased incidence of Parkinson's disease (Falck et al. 1992; Wolff et al. 1993; Faroon et al. 2001; Flower et al. 2004; Ascherio et al. 2006).

For public health safety and environment protection, various measures have been taken with concomitant investments of resources by government in most countries, but remediation of contaminated sites still remains a serious problem to be addressed. Various physicochemical processes including soil washing, electrokinetic methods, chemical reduction or oxidation of contaminant, incinerations, etc., appear to be expensive, environment invasive, and many times nonspecific, producing secondary contaminants (Pandey et al. 2009; Weyens et al. 2009, 2010a). Thus, there is a constant thrust to develop alternative strategies for remediating contaminated sites. During the last two decades, phytoremediation has emerged as a potential tool for the cleanup of polluted environment. Phytoremediation is the use of plants to remediate polluted soils which is an eco-friendly and cost-effective technology and currently receiving considerable global attention (Glick 2010; Yousaf et al. 2011; Khan et al. 2013). It is an *in situ* solar-powered remediation technology, requiring minimal site disturbance and maintenance resulting in high public acceptance (Weyens et al. 2009, 2010a). Since conventional remediation options currently available are frequently expensive and environmentally invasive, phytoremediation emerges out to be a valuable alternative, especially for the treatment of large contaminated areas with diffused pollution (Weyens et al. 2010a, b; Nesterenko-Malkovskaya et al. 2012).

Rhizoremediation: An Edge Over Phytoremediation

Plant uptake is the first and crucial step in whole plant metabolism of organics. In case of constant plant and environmental features, the lipophilicity of the compound, expressed as its octanol-water partition coefficient (K_{ow}), is the determining factor for root entry and translocation. Organic contaminants with a log K_{ow} <1 are considered to be very water soluble, and plant roots do not generally accumulate them at a rate surpassing passive influx into the transpiration stream (Cunningham and Berti 1993). Contaminants with a log K_{ow} >3.5 show high sorption to the roots but slow or no translocation to the stems and leaves (Trapp et al. 2001). However, plants readily take up organic contaminants with a log K_{ow} between 0.5 and 3.5, as well as weak electrolytes. After plant uptake, the organic compound may be metabolized and/

Table 4.1 Persistent organic pollutants of major concern enlisted by the Stockholm Convention 2004

POP	Use	Structure
Aldrin	Crop insecticide (corn, cotton)	
Chlordane	Crop insecticide (vegetables, citrus, cotton, potatoes)	
DDT (dichloro-diphenyl-trichloroethane)	**Crop insecticide (cotton)**	
Dieldrin	Crop insecticide (cotton, corn)	
Endrin	Crop insecticide (cotton, grains)	
Heptachlor	Insecticide (termites and soil insects)	
Hexachlorobenzene	Fungicide for seed treatment	
Mirex	Insecticide (termites, fire ants)	
Toxaphene	Insecticide (livestock, crops)	
PCBs	**Industrial chemical (paint and plastic additive)**	
Dioxins	**Unintentionally produced during combustion**	
Furans	Unintentionally produced during combustion	

or released into the atmosphere via evapotranspiration through the stem and/or leaves. Although plants often metabolize or sequester organics, they have significant inabilities in two ways, as being photoautotrophic; plants do not rely on organic molecules as a source of energy or carbon. By consequence, unlike microorganisms, during evolution plants were not under selective pressure to develop the capacity to degrade chemically recalcitrant molecules, leading to a much more limited spectrum of chemical structures that they can metabolize (Burken 2004). To avoid accumulation and potential toxicity to sensitive organelles, plant metabolizes organic molecules (other than photosynthates), converts them into more water-soluble forms, and sequesters into vacuole or cell walls. The process is described as the "Green Liver Model" (Sandermann 1992). By contrast, microbial metabolism often ends with the organics being converted into CO_2, water, and cellular biomass. Therefore, in order to obtain a more efficient degradation of organic compounds, plants depend on their associated microorganisms. Plants themselves have a positive effect on the microbial degradation of

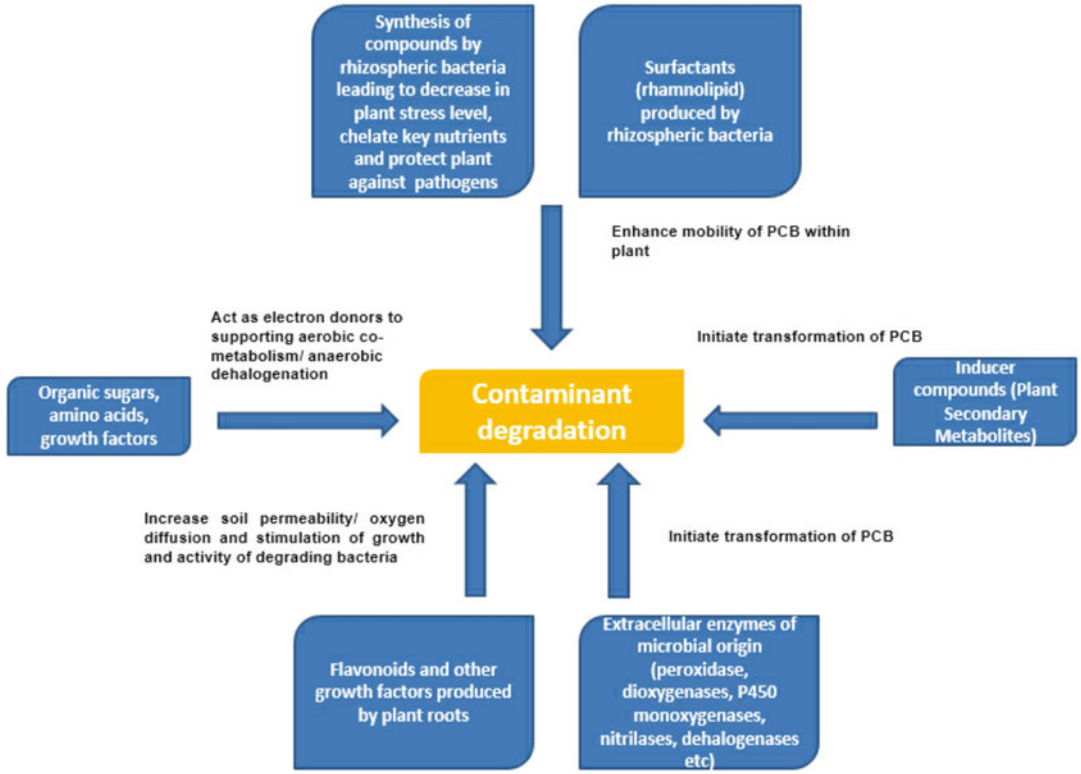

Synthesis of compounds by rhizospheric bacteria leading to decrease in plant stress level, chelate key nutrients and protect plant against pathogens
Surfactants (rhamnolipid) produced by rhizospheric bacteria
Enhance mobility of PCB within plant
Act as electron donors to supporting aerobic co-metabolism/ anaerobic dehalogenation
Initiate transformation of PCB
Organic sugars, amino acids, growth factors
Contaminant degradation
Inducer compounds (Plant Secondary Metabolites)
Increase soil permeability/ oxygen diffusion and stimulation of growth and activity of degrading bacteria
Initiate transformation of PCB
Flavonoids and other growth factors produced by plant roots
Extracellular enzymes of microbial origin (peroxidase, dioxygenases, P450 monoxygenases, nitrilases, dehalogenases etc)

Fig. 4.1 Synergistic effect of plant and rhizospheric microbes during rhizoremediation and phytoremediation

organic contaminants (Crowley et al. 1997). This increased degradation potential is the result of higher microbial densities and metabolic activities in the rhizosphere due to microbial growth on root exudates and cell debris originating from the plant roots. Some of the synergistic activities are summarized in Fig. 4.1. Moreover, dense populations of diverse heterotrophic microorganisms are living in the rhizosphere, phyllosphere, and inside the plant (endophytes). These microbial associations increase the capacity for a stepwise transformation of organic contaminants by consortia and provide a habitat that is conducive to genetic exchange and gene rearrangements. The emerging picture suggests that plants draw pollutants, including PAHs, into their rhizosphere to varying extents via the transpiration stream (Harvey et al. 2002). Subsequent degradation can occur in the plant itself, or in the rhizosphere, or both. However, plants readily absorb compounds with a log K_{ow} between 0.5 and 3.5. These compounds seem to enter the xylem faster so that rhizosphere microflora cannot degrade them, even if the latter is enriched with bacteria capable of degrading the compound (Trapp et al. 2000). Therefore, after this class of compounds is taken up by the plants, endophytes seem to be especially suitable for the degradation of these compounds. Endophytic bacteria can be defined as bacteria colonizing the internal tissues of plants without showing symptoms of infection or negative effects on their host, and of the nearly 300,000 plant species that exist on the earth, each individual plant is host to one or more endophytes (Strobel et al. 2004). The association of endophytes with their host is varied and complex, and we have just started understanding these interactions. Several plant growth promotory effects of endophytes on their host ranging from N_2 fixation, biocontrol against phytopathogens, phytohormone production, etc., are well

documented (Parmeela and Johri 2004; Compant et al. 2010; Gupta et al. 2013). The combined use of plants and endophytic bacteria to clean up environmental pollutants has gained momentum in the past years as they have high potential to promote plant growth and contribute to degradation of pollutants (Germaine et al. 2006; Compant et al. 2010; Khan et al. 2013).

Large-scale applications of phytoremediation still face a number of obstacles, including the levels of contaminants (being toxic for the organisms involved in remediation), bioavailable fraction of the contaminants (being too low), and, in some cases, evapotranspiration of volatile organic pollutants from soil or groundwater to the atmosphere. Exclusively derived processes by plants alone (phytoremediation) are time-consuming. Further, high levels of pollutants pose toxicity to the remediating plants. This process can be accelerated by exploring the plant-microbe partnership, which would improve the plant growth by facilitating the sequestration of contaminants modified by microbial communities in rhizosphere, which is more precisely called as plant-assisted rhizoremediation (Gerhardt et al. 2009; Afzal et al. 2011; Teng et al. 2011; Yousaf et al. 2011). Plant root systems efficiently absorb surrounding water, hence bringing dissolved pollutants into the rhizosphere zone for degradation. Contaminant-degrading bacteria can be conveniently included in plant growth-promoting rhizosphere bacteria as they colonize root vicinity and utilize root exudates as sole carbon and energy source facilitating concomitant degradation of organic contaminants (Singer et al. 2003). Also, plants positively affect the size of colonizing populations and exhibit certain preferences towards particular group/genera of microbial community. This preference is usually induced by shifts in chemical composition of root exudations (Jha et al. 2014). This strategy is referred as ex planta means of phytoremediation. Interestingly, plant roots not only secrete enzymes but also improve the degrading ability of microorganisms present in rhizo-/endorhizosphere (Gianfreda and Rao 2004; Kuiper et al. 2004).

Aminocyclopropane-1-Carboxylic Acid (ACC) Deaminase-Producing Bacteria and Phytoremediation

Roots are the most important part of a plant. The extensive root system not only provides anchorage but also helps in absorption of essential nutrients from soil so that the plant may flourish well. Sometimes roots play a larger-than-life role by hyperaccumulating metals and other organic contaminants from rhizosphere, thus, participating in phytoremediation. However, the stress induced due to the presence of contaminants exerts negative effect on plants by elevating ethylene production, a phytohormone known to reduce root growth, thus, posing a major limitation to phytoremediation. The phytohormone ethylene (C_2H_4) has a central role in modulating the growth and cellular metabolism of plants. This is believed to be involved in disease resistance, biotic/abiotic stress tolerance, plant-microbe partnerships, and plant nutrient cycle. The enhanced level of ethylene is usually plant's response to stress induced by the presence of metals, organic and inorganic chemicals, damage (mainly by insects and nematodes) or mechanical wounds, and most importantly, presence of phytopathogens (as bacteria and fungi) (Abeles et al. 1992). The synthesis of "stress ethylene" occurs in two peaks (Glick et al. 2007; van Loon et al. 2006). The first peak is relatively small and consumes the existing 1-aminocyclopropane-1-carboxylic acid (ACC), the immediate precursor of ethylene. This consumption of ACC in stressed plants is known to initiate the transcription of spectrum of genes responsible for encoding plant defensive proteins (Robinson et al. 2001). The second peak of ethylene in plant occurs by the synthesis of additional ACC in plant, and it is known to be detrimental to the plant. The overproduction of ethylene can cause the inhibition of root elongation and lateral root growth, and root hair formation consequently reduced phytoremediation efficiency. However, some bacteria are capable of alleviating the stress-mediated impact on plants by enzymatic hydrolysis of ACC. ACC is involved in the biosynthetic pathway of ethylene, as an intermediate in the

conversion of methionine to ethylene following biosynthetic sequence: methionine-S-adenosylmethionine (SAM)–ACC– C_2H_4 (Adam and Young 1979). In general, ACC is exuded from plant roots or seeds and then taken up by the ACC-utilizing bacteria before being oxidized by the plant ACC oxidase and cleaved by ACC deaminase to α-ketobutyrate (αKB) and ammonia. The bacteria utilize the ammonia evolved from ACC as a sole nitrogen source and thereby decrease ACC within the plant with the concomitant reduction of plant ethylene. The decreased ethylene levels in plants hosting ACC-utilizing bacteria derive benefits by stress alleviation and enhanced plant productivity. Since SAM is converted by ACC synthase to ACC, the ACC synthase protein seems to play a main controlling role in the ethylene biosynthesis pathway. In the absence of ACC-utilizing bacteria, ACC is oxidized by ACC oxidase to form ethylene, cyanide, and CO_2.

Plant: Microbe Partnerships for Enhanced Phytoremediation of Organic Pollutants

In order to understand, optimize, and monitor bio/rhizoremediation, a process occurring naturally in soil ecosystems, it is most important to explore and understand specific plant-microbe pairs which carry out these degradations efficiently in nature. Several plant species including *Salix caprea, Willow, Birch, Pinus, Poplus, Solanum nigrum, Nicotiana tabacum, Armoracia rusticana,* among others, are well known to harbor bacterial populations capable of degrading organic compounds especially PCBs (Leigh et al. 2007; Ionescu et al. 2009; Uhlik et al. 2012). Siciliano et al. (2001) showed that plants growing in xenobiotic contaminated soils have natural ability to recruit endophytes having catabolic genes (Siciliano et al. 2001). In a groundbreaking study, Leigh and coworkers (2006) investigated abundances, identities, and degradation capabilities of microbial populations associated with mature tree species (*Pinus nigra, Salix caprea*) naturally growing in PCB-contaminated sites and identification of plants that are capable of preferentially promoting growth of PCB-degrading bacteria in their rhizosphere. Authors found that plant species like *P. nigra* and *S. caprea* were having good rhizoremediation potentials as they were harboring increased number of PCB degraders in their root zones (Leigh et al. 2006). Both plants produced terpenoids, tannins, some phenolic compounds, among others, bearing marked analogy with aromatic pollutants that serve as a substrate and induced PCB degradation pathway (Singer et al. 2003). In another study conducted by Ionescu and coworkers (2009), four different plant species, viz., *S. caprea*, *S. nigrum, N. tabacum*, and *A. rusticana* were used to study the influence of plant growth on total microbial counts and plant-mediated effects on total counts of bacteria. Authors found that the most efficient plant species promoting the growth of PCB degraders were *N. tabacum* and *A. rusticana*. It is also proven experimentally that mixed cropping helps in inducing enzyme systems involved in the degradation of pollutants. Li et al. (2013) conducted an experiment to see the effect of mixed plantation on PCB removal mediated by two well-known plant species (alfalfa and tall fescue). Authors concluded that even mixed plantation contributes to significant increase in phytoremediation activity of associated microbial populations. Most common PCB degraders identified were *Pseudomonas mendocina*, *Sphingobacterium mizutae*, *Agrobacterium radiobacter*, *Ochrobactrum anthropi*, and *Burkholderia cepacia*. Other plants as *Festuca arundinacea, Morus* sp., *Panicum virgatum (switchgrass), Cucurbita pepo*, *Arabidopsis thaliana*, *Lolium multiflorum*, etc., are also well known as home to several PCB and other aromatic hydrocarbon-degrading microorganisms (Li et al. 2013). Some commonly used plant and bacteria combinations used successfully by various authors has been complied in (Table 4.2).

Rhizoengineering: Manipulating Root Exudations

Plants actively participate in recruiting or exhibiting preferences towards a specialized group of population to colonize its rhizosphere. This microbial community is often referred as the

Table 4.2 Plant-microbe combinations exploited in PCB degradation

Plant used	Microbes used	Origin	Reference
Medicago sativa	*Pseudomonas*	Rhizosphere	Ionescu et al. (2009)
	Rhodococcus		
	Rhizobium		
N. tabacum	*Achromobacter* sp.	Rhizosphere	Ionescu et al. (2009)
S. caprea	*Sphingobacterium mizutae*	Rhizosphere	Ionescu et al. (2009)
	Burkholderia cepacia		
A. rusticana	*Bacillus pumilus*	Rhizosphere	Ionescu et al. (2009)
S. nigrum	*Pseudomonas mendocina*	Rhizosphere	Ionescu et al. (2009)
	P. fluorescens		
Populus	*Burkholderia cepacia*	Endophytic	Taghavi et al. (2005)
Triticum spp.	*Herbaspirillum* sp. K1	Endophytic	–
P. nigra	*Microbacterium oxydans* type strain	Rhizosphere	Siciliano and Germida (1998)
S. caprea	*Achromobacter* sp.	Rhizosphere	Leigh et al. (2006)
Fraxinus excelsior	*Rhodococcus erythreus* type strain	Rhizosphere root zone	Schell (1985)
Betula pendula	*R. erythreus* type strain	Rhizosphere root zone	Schell (1985)
Robinia pseudoacacia	*Rhodococcus* sp., *Arthrobacter oxydans*	Rhizosphere root zone	Schell (1985)

second genome of plant (Berendsen et al. 2012). The vast spectrum of structural and functional diversities of plant root exudates and secondary plant metabolites (SPMs) is an eternal source to an interdisciplinary researcher to explore the dynamic microcosm of the rhizosphere. Hence, it has been envisaged as an ideal site to modify microbial populations to suit various applications in the soil. This manipulation of plant root secretions and associated microbes to improve plant health and productivity is termed as rhizoengineering (O'Connell et al. 1996). Rhizoengineering exploits the strategies based on favoring the growth of the targeted microbes that possess the ability to metabolize exotic nutrients exuded by plants (Lugtenberg et al. 2001). A nutritional bias or favoritism is created, and population of bacteria can be further enriched which can preferentially utilize the given nutrients. Savka and Farrand (1997) published an early report to achieve success in rhizoengineering based on favorably partitioning the exotic nutrients, opines, which were produced by the transgenic plants leading to improved and competitive growth of the metabolizing strains in comparison with the microbes unable to metabolize opines (opine concept). This strategy provides selective nutritional advantage to *Agrobacterium* and other selective bacteria considering genetic exchanges (horizontal gene transfer). In yet another approach, various species of *Pseudomonas* selectively using salicylate as carbon source (Colbert et al. 1993) and detergents (Igepal Co-720 and dioctyl sulfosuccinate) from maize rhizosphere had been isolated. Flavonoids, most studied as chemicals involved in molecular cross talk between plant roots and specific microorganisms, are known to induce transcription of nodulation genes in nitrogen-fixing rhizobia (Brencic and Winans 2005). *Rhizobium* sp. and *Bradyrhizobium* sp. transformed the flavonoids quercetin, daidzein, and genistein through novel biochemical pathways in contrast to modes of degradation favored by other rhizobacteria (Toussaint et al. 2012). Flavonoids, naringenin, and apigenin have been shown to support growth of PCB-degrading bacteria (Fletcher and Hegde 1995). Plant production of substrates that cause nutritional bias on the one hand and enzyme induction for the biodegradation of xenobiotic compounds on the other offers intriguing and very attractive opportunities for rhizosphere manipulations (rhizoengineering) for enhancing rhizodegradation of xenobiotic contaminants beyond the inherent capabilities of the plant-microbe system. Further researches focused on profiling of root exudates

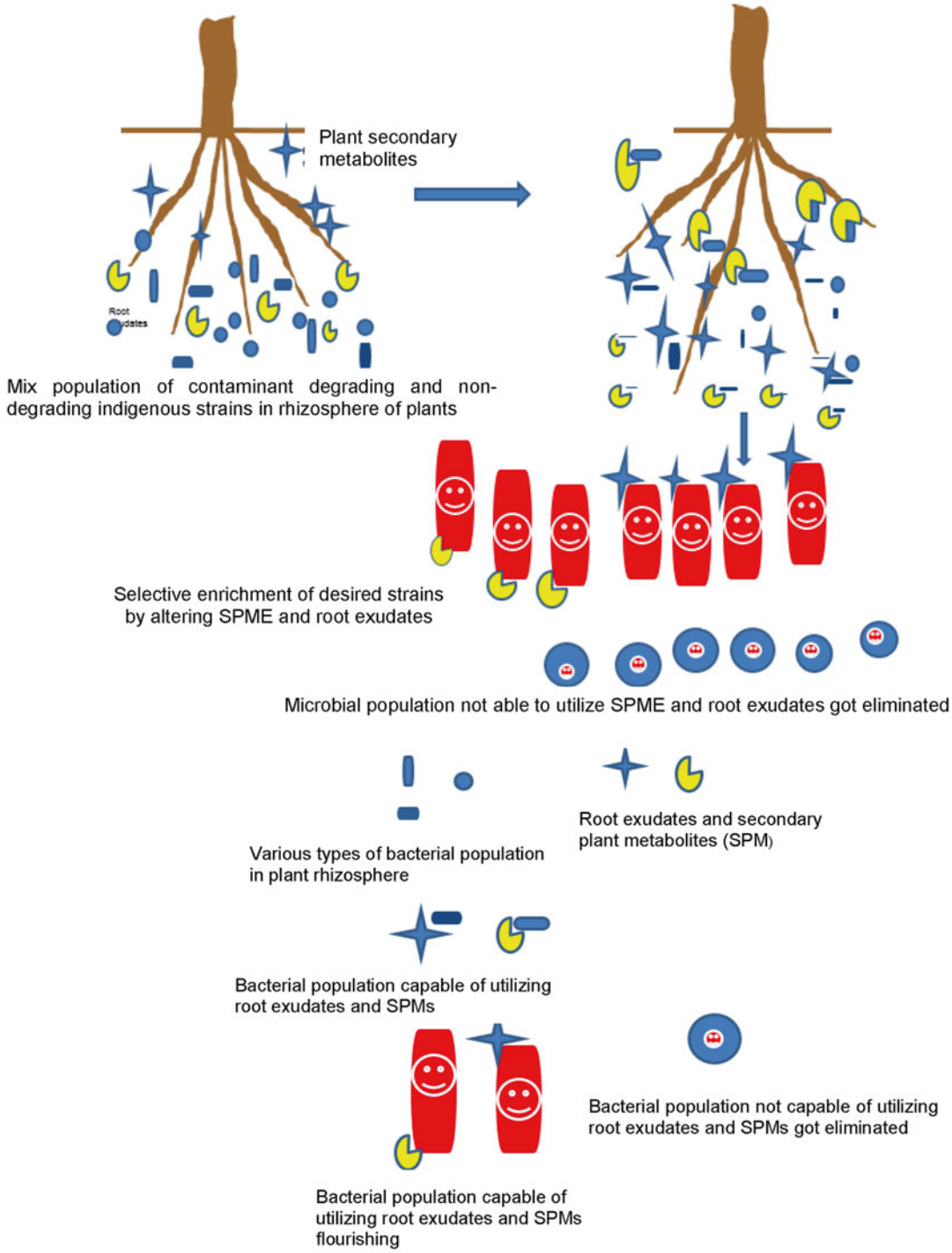

Plant secondary metabolites
Root exudates
Mix population of contaminant degrading and non-degrading indigenous strains in rhizosphere of plants
Selective enrichment of desired strains by altering SPME and root exudates
Microbial population not able to utilize SPME and root exudates got eliminated
Various types of bacterial population in plant rhizosphere
Root exudates and secondary plant metabolites (SPM)
Bacterial population capable of utilizing root exudates and SPMs
Bacterial population not capable of utilizing root exudates and SPMs got eliminated
Bacterial population capable of utilizing root exudates and SPMs flourishing

Fig. 4.2 Role of root exudates and SPMs in contaminant (polychlorinated biphenyls, polyaromatic hydrocarbons) degradation

to identify compounds that may be exploited to create nutritional bias for enhancing rhizodegradation of persistent xenobiotic contaminants such as PCB have immense potential (Narasimhan et al. 2003). A schematic representation of rhizoengineering is demonstrated in Fig. 4.2.

Approaches to Study Diversity and Activity of Microbes in Contaminated Soil

The advents of molecular biology techniques bypassing cultivation steps have enabled us to generate information on the total microbial composition at a particular site and time (Table 4.3). These molecular phylogenetic techniques are based on isolating DNA directly from environmental samples as soil, water, sediments, etc. However, there are certain advantages and disadvantages associated with each culture-dependent and culture-independent techniques. Few important techniques currently being used are discussed in brief here.

Biochemical Identification

It represents one of the oldest means of microbial identification and characterization. Several commercial kits are available in facilitating easy and rapid identification of pure cultures on the basis of phenotypes (Busse et al. 1996). NEFERM test 24, API 20, BIOLOG plates, among others, are most routinely used in microbiological laboratories. However, identification based on biochemical tests suffers major drawbacks of being labor intensive, requirement for specific consumables, and having lengthy processes.

Whole-Cell MALDI-TOF MS

This is relatively a recent and revolutionary technique, providing accurate measurement of molecular weight of a compound and giving information on molecules and its structure. Difference in mobility of current carriers with different molecular weight under electromagnetic field is the main principle for this technique (Lay 2000). Fragments of proteins, higher molecular weight fragments of lipid oligosaccharide components of the cell wall, serve as ionized molecules and biomarkers conserved within a particular group of microorganisms and, hence, allow identification, easy grouping, and characterization. Kurzawova et al. (2012) successfully used the technique to identify bacterial species based on the composition of ribosomal proteins in bacterial cells isolated from two plant species *N. tabacum* and *S nigrum*, well known to house PCB degraders. The technique is quick and requires less effort in sample preparation. Relatively low sample size requirement makes it popular among research fraternity (Krishnamurthy and Ross 1996). In a recent variation of the technique, whole-cell instead of extracted proteins or other molecules from environmental samples is used. Koubek et al. (2012) in an interesting study compared three different methods, viz., NEFERM test 24, MALDI-TOF MS, and sequencing of 16S rRNA for taxonomic identification of bacterial isolates recovered from sediments contaminated with PCB. Authors found that each method has its own limitation and advantage for resolution. However, MALDI-TOF MS appeared as the most reliable method with immense potential for quick screening of clinical and environmental samples (Koubek et al. 2012). Restriction patterns of PCR-amplified fragments of particular gene can also be analyzed with MALDI-TOF MS (Taranenko et al. 2002).

Genetic Fingerprinting Techniques

Genetic fingerprinting techniques provide a pattern of genetic diversity in a microbial community. There are several fingerprinting techniques including denaturing gradient gel electrophoresis (DGGE)/thermal gradient gel electrophoresis (TGGE), terminal restriction fragment length polymorphism (T-RFLP), amplified ribosomal DNA restriction analysis (ARDRA), DNA sequencing, and real-time PCR among others. A genetic fingerprinting technique is based on

Table 4.3 Tools and techniques used to identify microorganisms metabolizing various organic pollutants

Technique	Environmental sample	Target compound	Microorganism recovered	Reference
Biochemical tests	Sediment	PCB	*P. fluorescens*	Dercova et al. (2008)
			P. aeuroginosa	
			Ochrobactrum anthropi	
			A. radiobacter	
Mass spectrometry (MALDI-TOF)	Horseradish rhizosphere soil	Biphenyl	*Rhizobium radiobacter*	Uhlik et al. (2011)
			Arthrobacter sp.	
			Achromobacter sp.	
			Rhodococcus sp.	
			Serratia fonticola	
16S rDNA sequencing	Alfalfa and tall fescue rhizosphere soil	PCB	*Burkholderia* sp.	Li et al. (2013)
			Pseudomonas sp.	
			Comamonas testosteroni	
DGGE/TGGE	Soil	PCB	*Flexibacter* sp.	Tang et al. (2013)
			Bdellovibrio sp.	
			Bacillus niabensis *Bradyrhizobium elkanii*	
			Sphingomonas sp.	
T-RFLP	Soil	Chlorobiphenyl	*Hydrogenophaga*	Uhlik et al. (2013)
			Pseudoxanthomonas	
			Azoarcus	
			Burkholderia	
SIP	Soil	Biphenyl	*Burkholderia*	Uhlik et al. (2012)
			Pseudomonas	
			Rhodanobacter	
SIP	Soil	Benzoate	*Rhodanobacter*	Uhlik et al. (2012)
			Pseudomonas	
			Azotobacter	
SIP	Soil	Naphthalene	*Pseudomonas*	Uhlik et al. (2012)
			Rhodanobacter	
			Pandoraea	
SIP	Soil	Chlorobiphenyl		Uhlik et al. (2013)

deducing polymorphism in total or a part of genetic material. DGGE is based on the identification of differences in base sequences even if the size of DNA fragments is the same. The principle behind the technique is decreased electrophoretic mobility of DNA in polyacrylamide gels containing a linear gradient of DNA denaturants (DGGE) or a linear temperature gradient (TGGE). In both DGGE and TGGE, DNA is PCR-amplified with primers targeting part of the studied gene, mostly 16S rRNA gene or some specific catabolic activity gene. The 5′ of the forward primer is attached with 35–40 base pair GC clamp which facilitates a certain portion of the DNA to remain double stranded during electrophoresis. In the following denaturation step, hydrogen bonds are disrupted while holding together double strands of DNA (Marsh and Nakatsu 2007). Hence, the separation of DNA occurs proportional to G + C content and distribution. Watanabe and coworkers beautifully exploited the technique to detect and characterize dominant phenol-degrading bacteria in activated sludge (Watanabe et al. 1998). In a similar technique, single-strand conformational polymorphism (SSCP), the separation of single-stranded

DNA is achieved on the polyacrylamide gel and differences in mobility results due to differences in the folded secondary structure. Here no denaturants are used; hence, DNA molecules of equal size fold and hence move differently according to the DNA base sequences (Schiwieger and Tebbe 1998). Ribosomal intergenic spacer analysis (RISA) is yet another technique being exploited in microbial community analysis of a contaminated site. The heterogeneity of length and sequences of tRNAs encoded in intergenic spacer region between 16S and 23S rRNA genes is studied for differentiation between bacterial strains and closely related species. Again, in RISA, the intergenic spacer region is PCR-amplified, denatured, and separated on polyacrylamide gel. The sequence polymorphisms are detected by silver stain or in an advanced version, i.e., automated ribosomal intergenic spacer analysis (ARISA), and the forward primer is fluorescently labeled and is automatically detected, which increases the sensitivity of the method. In an interesting study, Watts and coworkers (2001) while studying PCB-dechlorinating communities in enrichment culture did comparative analysis among three molecular techniques ARDRA, DGGE, and T-RFLP. Authors found that the methods have different biases as the same enrichment culture gave different clone frequencies (ARDRA), band intensities (DGGE), and peak heights (T-RFLP). However, all the techniques proved effective in qualitative analysis and identification of organism (Watts et al. 2001). Also, authors indicated that of all the techniques DGGE appeared to be rapid and the most effective tool for studying microorganisms in highly enriched cultures.

Stable Isotope Probing (SIP)

This is a relatively new technique that relates microbial diversity to the metabolic activity, initially reported by Boschker et al. (1998) to investigate oxidation of greenhouse gas methane in freshwater sediment (Boschker et al. 1998). There exist several variations including DNA-SIP, RNA-SIP, SIP of amino sugars, and SIP of methyl esters of fatty acids (PLFA-SIP). SIP directly allows tracking of stable isotope (C^{13}) from an environmental sample in order to probe which microbes are functionally active in utilizing a given pollutant. This is done usually through incubating a sample with a heavier isotope of carbon-, nitrogen-, or oxygen-labeled compound. This compound is usually a substrate for that community. The labeled compound if utilized is inserted into the cellular components after which phylogenetic markers as DNA, RNA, phospholipid fatty acids, or proteins can be extracted and analyzed. Uhlik et al. (2012) in an interesting study investigated shifts in bacterial community composition induced by naringenin, caffeic acid, and limonene, due to their degradative capabilities on long-term PCB-contaminated soil. They coupled pyrosequencing of 16s rRNA gene tag-encoded amplicons and DNA-stable isotope probing (SIP) to analyze and identify populations actively involved in 4-chlorobiphenyl catabolism. Authors concluded that out of three PSMs used, application of naringin showed efficient degradation when compared to the control. Also, they identified that *Hydrogenophaga*, *Terrimonas*, *Paucimonas*, and *Pseudorhodoferax* were the most dominant genera.

Radajewski et al. (2000) enhanced the phylogenetic resolution of SIP by demonstrating that stable isotope-labeled DNA could be used to identify individual members of mixed microbial communities. Since isotopic enrichment increases buoyant density of DNA, subsequent density centrifugation in CsCl gradients is exploited to separate "heavy" (labeled) from "natural" (unlabeled) DNA. As a final step 16S rDNA clone libraries are constructed from "heavy" DNA and sequenced to obtain the identity of organisms assimilating the given ^{13}C-labeled substrate used. Another major variation of the technique is RNA-SIP where SSU (small subunit) rRNA molecule can be used (Manefield et al. 2002). Huang et al. (2009) successfully used mRNA-SIP to recover the key phylogenetics and functional RNAs and concluded that RNA-SIP can be used for studying transcriptomes together with taxonomic identities (Huang et al. 2009). However, there are certain serious shortcomings to the tool, being low

mRNA yields from environmental samples, difficulties in separating it from other types of RNAs, and its relative instability.

Biosensors

Biosensors can be defined as a coupling of any biological system with microelectronic or any other device which facilitates the rapid, accurate, and detection of very low levels of contaminants in body fluids, water, or soil. Biosensors based on microorganisms are emerging as candidates of choice being easy to maintain, cost-effective, high population sizes, and accurate in activity and functioning. Additionally, they can be tailored to meet the specific requirements when monitoring specific contaminants. Casavant et al. (2003) used a *gfp*-labeled *Pseudomonas fluorescens* A506 to detect low concentrations of toluene (0.2 mm) and trichloroethylene in the rhizosphere and observed an increased *gfp* expression in the root-colonizing biosensor population when the plant rhizosphere was exposed to toluene. Interestingly, they noted that 14 % more cells were induced in uncontaminated rhizosphere soil than bulk soil. It was further confirmed that the promoter is induced by variety of alkyl-substituted benzene derivatives and branched alkenes (Singer et al. 2003).

Biosensors also appear to be useful tools when carrying out environmental risk assessments of polluted sites while monitoring the efficacy of a remediation strategy because of their ability to detect only the biologically relevant (bioavailable) fraction of the contaminant. Liu et al. (2010) in a study constructed three biosensor strains: *P. fluorescens* F113rif*gfp*, *P. fluorescens* F113rifPCB*gfp*, and *P. fluorescens* F113L::1180*gfp* designed to detect the bioavailability and biodegradation of PCBs in real contaminated soil. As the F113rifPCB*gf*p and F113L::1180*gfp* strains degraded PCBs, they produced chlorinated benzoate end products which in turn induced the expression of *gfp*. Therefore, these strains could actively report degradation of PCBs by their own due to the fluorescence in the presence of an end product (benzoate). This approach is equally suited to study both soil bioremediation and plant rhizoremediation. Some authors suggested that by immobilizing the biosensor cells, ease of detection, accuracy, and reproducibility could be improved. There exists a positive correlation between the PCB levels within the samples and the percentage of fluorescing biosensors cells when immobilized PCB-degrading biosensors (F113L::1180*gfp* and F113rifPCB*gfp*) were introduced into PCB-contaminated soil and sludge. The greater number of fluorescent cells is a direct manifestation of greater bioavailability of PCBs, and their degradation by the GM (genetically modified) biosensors which in turn indicated that the soil/sludge posed a greater risk to human health. Demnerova et al. (2005) carried out PCB degradation experiments using three different plant species. Their results supported previous existing plethora of data that the number of natural PCB degraders on roots was at least ten times higher than that in bulk soil. Gfp-expressing biosensor cells were easy to detect, and they were in higher densities in soils planted with pea, indicating increased PCB degradation activity compared with unplanted soil. Authors stated that this study demonstrated that biosensors could be used at two major fronts, firstly, to evaluate a specific contaminated site for bioremediation potential, and secondly to monitor PCB degradation in real PCB-contaminated soil.

qRT-PCR

Use of real-time PCR increases the probability of accurately determining population sizes of PCB-degrading microbes. Biosensor cell numbers detected by the qRT-PCR method are tenfold higher than the numbers detected using the plate counting method. The high sensitivity of real-time PCR is because it could detect both culturable and non-culturable inoculants together with dead inoculants (Cubero and Graham 2005). It was also documented previously by Wang et al. (2004) that by using a qRT-PCR method, a tenfold higher level of the engineered *Pseudomonas putida* could be detected compared to the plate counting method, during 2-chlorobenzoate degradation in soil.

Now next-generation sequencing, such as pyrosequencing along with various bioinformatics software tools, is emerging as a potential tool for identification of functional communities of microbes present in polluted sites.

Conclusion

Rhizoremediation has successfully occupied center stage in the remediation of sites contaminated by metals and especially organic pollutants. Several genera of bacteria have already proved their potentials in lowering the levels of organic pollutants along with their plant partner. Thus, it becomes immensely important to explore some new such partnerships to optimize remediation strategies in long-term contaminated soils. Recently, molecular biology-based techniques have broadened our scope in not only identifying the phylogeny of the individual members in bacterial communities engaged in polluted sites but also in linking their phylogeny with metagenomics. Thus, in order to fully understand and optimize the bioremediation processes, much deeper researches based on exploring diversity in metabolic pathways and understanding the mutual relationships between or among plants and their associated microbes should be of paramount importance to help achieve goals. Use of biosensors, another complementary tool, has enabled us to have deeper insight in monitoring the efficiency of various environmentally oriented bioassays.

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Harnessing Plant-Microbe Interactions for Enhanced Protection Against Phytopathogens

5

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Abstract

Beneficial plant-microbe interactions have utmost importance for enhancing plant growth, improving soil structure, and managing plant diseases. Not surprisingly, such mutual interactions, where plants provide nourishment to rhizospheric microbes and in return microbes help in facilitating plant growth and stress amelioration, actually lay the foundation of sustainable agriculture. To cope with the major challenge of pathogen attack, beneficial rhizospheric microbes have proven their efficacy by induced systemic resistance (ISR). Therefore, such microbes are increasingly used in the form of biofertilizers and biopesticides. Moreover, such plant-microbe interactions elicit a range of defense-responsive activities in order to combat the pathogen challenge. The main microbes-mediated defense strategies adopted by plants include activation of antioxidant status of the plant by reprogramming defense-related enzymes, modulation of quorum sensing phenomenon, and activation of phenylpropanoid pathway leading to phenolics production, lignin deposition, and transgenerational defense response. In this chapter, we highlight the relevance of beneficial interactions between plant and microbes in enhancing plants' innate immune system against pathogen attack. This review provides a better understanding of the recent advances and major outcome of positive plant-microbe interactions and linking their relevance to plant defense response.

Introduction

Plants are the basis of life on earth that provide carbon source for all non-photosynthetic organisms. However, ~300,000 plant species are attacked by a huge number of detrimental organisms including pathogens and insects (Pieterse and Dicke 2007). Such biotic factors tremendously

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influence the plant growth and limit total agricultural production. Hence, sustainable approach of enhancing plant growth and managing plant diseases is being integrated to achieve higher crop yield. Plants in nature interact with wide range of beneficial and detrimental microorganisms providing baseline for linking aboveground and belowground community members (Van der Putten et al. 2001; Pineda et al. 2010).

The successful establishment of plant-microbe interaction depends on the ability of roots to interact with microbes as rhizosphere is the main zone where such interactions take place. The rhizospheric interaction could directly affect the plant growth by exerting either beneficial, neutral, or detrimental effects. Beneficial plant-microbe interactions require involvement of plant growth-promoting rhizobacteria (PGPR), endophytes, and mycorrhizal fungi that enhance plant growth by improving nutritional status for plants and helping the plants to combat abiotic and biotic stresses (Harrison 2005; Berendsen et al. 2012). In contrary, the detrimental interaction is imparted by pathogenic microorganisms resulting into various kinds of plant diseases. In the crowd of mixed population of beneficial and detrimental microbes residing in the rhizospheric region, the selection of potent biocontrol microbes is necessary to eliminate the pathogens and to combat the challenge imposed by pathogens. Moreover, plants also develop strategies spontaneously to recognize biotic and abiotic interactions and further translate the signal into defense response (Pei et al. 2000; Jones and Dangl 2006; Dicke and Hilker 2003).

Interestingly, plant-microbe interaction is regulated through signal-transduction pathways allowing plants to prioritize defense responses following stress conditions. It is well known that plants' response to pathogen attack is regulated by jasmonic acid (JA) and salicylic acid (SA)-dependent pathways effective against necrotrophic and biotrophic pathogens, respectively (Pieterse et al. 2012). The induced resistance imparted by these pathways represents two distinct responses: systemic acquired resistance (SAR) and ISR (van Loon et al. 1998). SAR is mediated by SA which is frequently produced following pathogen infection (Park and Kloepper 2000; Jeun et al. 2004). In contrary, JA- and ethylene (ET)-dependent ISRs are activated by beneficial microbes proven to produce antimicrobial compounds, siderophores, O-antigen of lipopolysaccharides (LPS) and salicylate. Moreover, ISR leads to the expression of pathogenesis-related (PR) proteins such as PR-1, PR-2, chitinases, and some peroxidases (POxs) (van Wees et al. 2000; Silva et al. 2004; Jetiyanon 2007). Considering the importance of ISR, recently, noteworthy consideration is being given to exploiting the beneficial soil microbes for enhancing plants' immunity against pathogen attack. Moreover, microbe-mediated suppression of plant diseases provides eco-friendly and sustainable approach of plant disease management. It is believed that such beneficial microbes can enhance the plant's innate immunity level against the invading pathogens by inducing an array of defense responses that include enhancement in antioxidant status of the plant by reprogramming defense-related enzymes, modulation of quorum sensing activities, and activation of phenylpropanoid pathway leading to phenolics production and lignin deposition.

Microbe-Mediated Antioxidants Status in the Host

Successful pathogen infection in plants results into oxidative burst that lead to production of reactive oxygen species (ROS) causing oxidative destruction of the cell (Asada 1999; Dat et al. 2000). These ROS are formed as a result of excitation of O_2 to singlet form of oxygen or via formation of a superoxide radical (O_2^-), hydrogen peroxide (H_2O_2), or a hydroxyl radical (HO^-). The enhanced production of ROS after pathogen infection acts as cellular indicator of stress conditions and is triggered by the activity of NADPH oxidases (Cazale et al. 1999; Pei et al. 2000). However, recently, it has been investigated that apart from NADPH oxidases, the other sources such as amine oxidases and cell wall-bound POxs also participate in the formation of ROS during programmed cell death and pathogen defense (Dat et al. 2000; Grant and Loake 2000). It is interesting to note that though the elevated level of ROS imposes threat condition to the cells, it also acts as a signal for commencement of stress

Table 5.1 Major ROS scavengers in plants

Scavengers	Localization	Target ROS
Superoxide dismutase	Chl, Cyt, Mit, Per, Apo	O_2^-
Ascorbate peroxidase	Chl, Cyt, Mit, Per, Apo	H_2O_2
Catalase	Per	H_2O_2
Glutathione peroxidase	Cyt	H_2O_2, ROOH
Peroxidases	CW, Cyt, Vac	H_2O_2
Thioredoxin peroxidase	Chl, Cyt, Mit	H_2O_2
Ascorbic acid	Chl, Cyt, Mit, Per, Apo	H_2O_2, O_2^-
Glutathione	Chl, Cyt, Mit, Per, Apo	H_2O_2
α-Tocopherol	Membranes	ROOH, O_2^1
Carotenoids	Chl	O_2^1

Apo apoplast, *Chl* chloroplast, *CW* cell wall, *Cyt* cytosol, *Mit* mitochondria, *Per* peroxisome, *Vac* vacuole

response and defense pathways (Desikan et al. 2001). The steady-state level of ROS in plant cells should always be under control as their over accumulation eventually result in cell death as a consequence from various kinds of oxidative processes, viz., lipid peroxidation, protein oxidation, nucleic acid damage, and enzyme inactivation.

In order to cope with the detrimental effects of elevated level of ROS, plants are bestowed with efficient antioxidant enzymes that contribute to ROS-scavenging mechanisms of plants. Major ROS scavengers in plants are reported to be superoxide dismutase (SOD), ascorbate peroxidase (APX), and catalase (CAT) (Mittler 2002) (Table 5.1). To maintain the balance of superoxide radicals in cells, the activities of SOD and APX or CAT enzymes play crucial role as this balance avert formation of hydroxyl radical via the metal-dependent Haber–Weiss or the Fenton reactions (Asada and Takahashi 1987; Bowler et al. 1991). Moreover, the key pathways of ROS scavengers in plants include SOD present in more or less all cellular compartments. The major threat to plant system is instigated by various kinds of plant pathogens which upon infection lead to rapid formation of ROS. Plants generally increase their tolerance against invading pathogens by elevating activities of antioxidant enzymes.

The major defense strategy adopted by plants is phenylpropanoid pathway catalyzing transformation of L-phenylalanine into trans-cinnamic acid (Dixon and Paiva 1995) which play an important role in the biosynthesis of phenolics having strong antimicrobial properties (Nicholson and Hammerschmidt 1992). Interestingly, activation of the phenylpropanoid pathway also leads to deposition of lignin and induction of antioxidant enzymes including SOD and peroxidase (POx) (Silva et al. 2004; Singhai et al. 2011). It is interesting to note that beneficial soil microflora in rhizosphere provide supportive environment for the plants by augmenting the antioxidant status. Such microbe-mediated ISR protects the plants from various plant pathogens. Earlier reports have revealed significant role of many microbes in enhancing antioxidant enzymes in plants contributing to their resistance against pathogens (Table 5.2). Previously, rhizobacterial strains and *Serratia marcescens* were reported to enhance activities of POx, phenylalanine ammonia lyase (PAL), polyphenol oxidase (PPO), and lipoxygenase (LOX) in betelvine and tomato after pathogen attack by *Phytophthora nicotianae* and *Pseudomonas syringae*, respectively. Modulation of such antioxidant status in plants treated with beneficial rhizobacteria enhanced disease resistance against pathogen attack (Diallo et al. 2011). Similar observation was recorded by Singhai et al. (2011) where pseudomonad strains were found to increase the level of POx and PAL in potato leading to tolerance against potato scab disease caused by *Streptomyces scabies*. Similarly, in another interesting study by Jain et al. (2012), the microbial consortium enhanced tolerance in pea plants against *Sclerotinia sclerotiorum* by inducing the level of PAL, POx, PPO, and SOD. Most recently, Singh et al. (2013) showed elevation of SOD and PO in *Sclerotium rolfsii* challenged chickpea plants treated with triple consortium of *Pseudomonas*, *Trichoderma*, and *Rhizobium*. Taken together, these reports clearly validate the importance of beneficial rhizosphere microbes in imparting tolerance to the plants against diverse range of pathogens by modulating their innate antioxidant status as shown in Fig. 5.1.

Table 5.2 Examples of microbe-mediated defense response in plants against pathogen infection

Host plant	Microbes	Antioxidant enhanced	Pathogen	References
Chickpea	*Pseudomonas*, *Trichoderma*, *Rhizobium*	Superoxide dismutase, peroxidase	*Sclerotium rolfsii*	Singh et al. (2013)
Pea	*Pseudomonas*, *Trichoderma harzianum*, *Bacillus subtilis*	Phenylalanine ammonia lyase, peroxidase, polyphenol oxidase, superoxide dismutase	*Sclerotinia sclerotiorum*	Jain et al. (2012)
Potato	Pseudomonad strains	Peroxidase, phenylalanine ammonia lyase	*Streptomyces scabies*	Singhai et al. (2011)
Betelvine	*Serratia marcescens*	Phenylalanine ammonia lyase, peroxidase, polyphenol oxidase	*Phytophthora nicotianae*	Lavania et al. (2006)
Tomato	Rhizobacterial strains	Lipoxygenase, phenylalanine ammonia lyase, peroxidase	*P. syringae* pv. *tomato*	Silva et al. (2004)

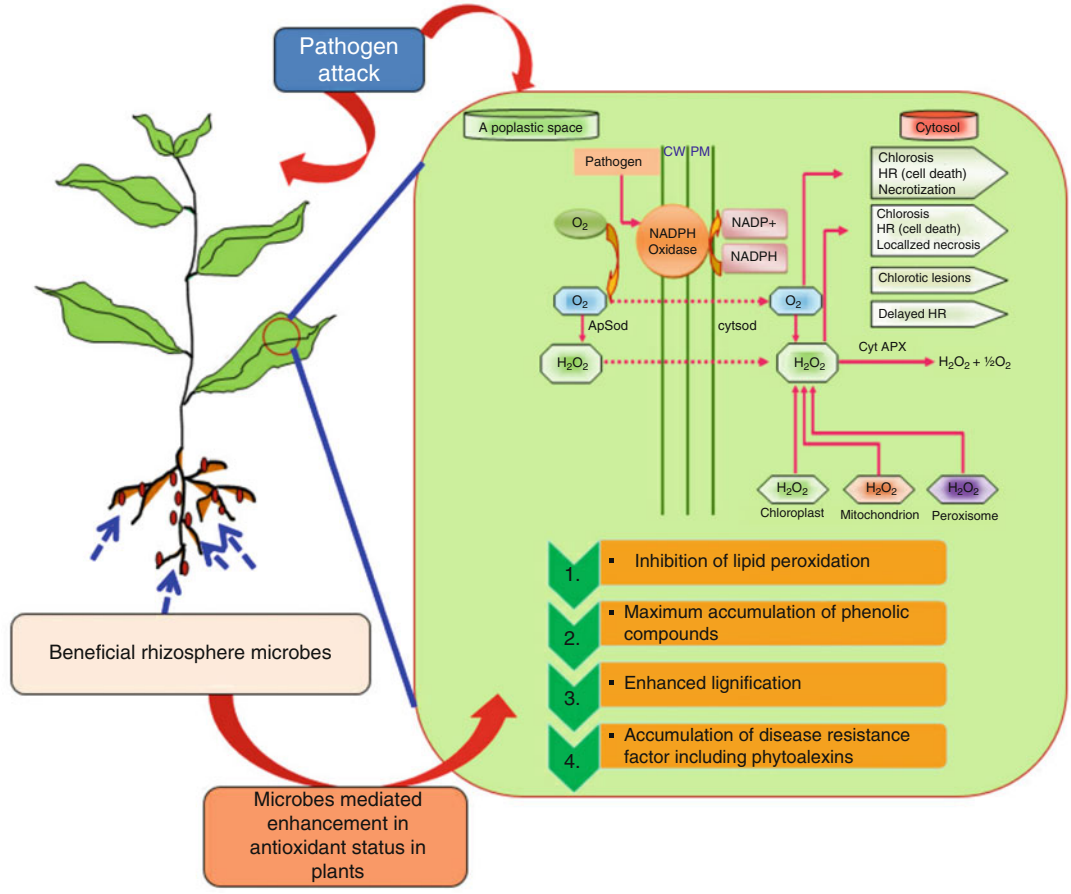

Fig. 5.1 Schematic diagram showing beneficial rhizosphere microbes mediated enhanced antioxidant activity contributing to their defense strategy against pathogen attack

Microbe-Mediated Activation of Phenylpropanoid Pathway

Soil is the locale of numerous microorganisms and can aptly be referred to as the mine of microorganisms. The rhizospheric regions of the plants inhabit most of the microbial communities in its vicinity, be it having beneficial or deleterious effects on the plant. Beneficial microorganisms may include PGPR and mycorrhizal fungi that induce systemic defense response in the host to preclude it from the chronic impairment of phytopathogens comprising of bacteria, fungi, viruses, and nematodes. To avoid the perplexity in the use of different terms for denoting the beneficial microbes, two new terms were proposed by Bashan and Holguin (1998): "biocontrol plant growth-promoting bacteria (PGPB)" that suppress plant diseases by enhancing the plant defense responses and "PGPB" that specifically play eminent role in augmenting plant growth. The upgradation of the conventionally used "PGPR" term coined by Kloepper et al. (1980) to "PGPB" would thereby include the useful microbes that do not inhabit the rhizospheric region of the soil yet enhance the vigor of plants against the phytopathogens.

Diverse interactions prevail between the microbial commune and plant roots, for instance, the symbiotic associations by mycorrhizal fungi that aid in the uptake of water and minerals (Harrison 2005), and the nodule-inhabiting *Rhizobium* bacteria that fix the atmospheric nitrogen for the plant (Spaink 2000). Several other types of beneficial microbes like PGPR and fungi are reported to suppress plant diseases (Van Loon et al. 1998; Harman et al. 2004; Kloepper et al. 2004) or insect herbivory (Van Oosten et al. 2008) by enhancing the defense response of the plant thereby resulting in overall increment in the plant growth parameters. The plethora of benefits endowed to the plants by the microbes can be attributed to either direct effect through mycoparasitism of soil-borne pathogens or indirectly by eliciting the plant defense mechanisms thereby fortifying the plant immune system against the invading pathogens (Van Loon et al. 1998; Pozo and Azcon-Aguilar 2007).

Several microbial determinants are related with the elicitation of defense responses in plant system. Most commonly studied and well associated as the inducers of host immune response conferred by rhizobacteria are the microbe-associated molecular patterns (MAMPs). Also, LPS and flagellin found at the cell surfaces of rhizobacteria are found to be potent in inducing defense response of the associated host against phytopathogens (Bakker et al. 2007). Another important mechanism employed by beneficial microbes in disease suppression is to create competition for iron by forming low molecular weight iron chelators known as siderophores (Meziane et al. 2005). Important chemicals that act as elicitors for the defense response are the variously secreted antibiotics by the rhizobacteria and fungi (Ran et al. 2005); surfactin, a lipoprotein secreted by *Bacillus subtilis* (Ongena et al. 2007); biosurfactant massetolide A from *P. fluorescens* (Tran et al. 2007); N-alkylated benzylamine (Ongena et al. 2005); N-acyl-L-homoserine lactone (Schuhegger et al. 2006); and volatile organic compound 2, 3-butanediol by *Bacillus* spp. (Ryu et al. 2004). Fungal proteins like endochitinase secreted by *Trichoderma* spp. have been shown to enhance plant defense-related proteins (Harman and Shoresh 2007; Keswani et al. 2014). A small protein SM1 produced by strains of *Trichoderma virens* can induce terpenoid phytoalexin biosynthesis and POx activity as studied in cotton plants and other systems as well (Djonovic et al. 2006, 2007). Another class of proteins associated with the immune system of the plants is the products of avirulence like (Avr) genes, produced not only by the phytopathogens but also by the beneficial microbes. They generally function as pathogen-specific elicitors of the hypersensitive responses in plants containing the corresponding resistance (R) gene (Woo et al. 2006). Small secondary metabolites produced by *Trichoderma* species have also been reported to possess defense response induction activity mainly via eliciting the expression of PR proteins on application to plants causing both local and

systemic disease suppression (Vinale et al. 2008; Keswani et al. 2014).

Plant defense response gets activated by the metabolism of phenyl propanoid (PP) pathway in which PAL catalyzes the first important step of the general PP metabolism. Further, the pathway leads to the synthesis of other important compounds having indispensible role in providing plant defense including cell wall strengthening and repair (lignin and suberin), antimicrobial activity (pterocarpan, isoflavonoid phytoalexins), and signaling SA (Hummerschmidt 1999). Beneficial microbes like *Trichoderma* spp. have been reported to trigger the terpenoid phytoalexin defense compounds in cotton seedlings thereby controlling *Rhizoctonia solani* infestation apart from the conventional mycoparasitic mode exhibited by the species of the genus (Howell et al. 2000). Also, biocontrol of *Pythium ultimum* on *Arabidopsis* seedlings by *T. harzianum* strain T22 reported the elevated expression level of *NPR1* gene which is the main gene involved in disease resistance (Shoresh et al. 2010). Recently, the concept of using two or three compatible beneficial microbes has been proposed for better management of disease. It has been shown that significant enhancement of PP activity is recorded in chickpea plants treated with the triple consortium developed using *Trichoderma*, *Bacillus*, and *Rhizobium* spp. when challenged with *S. rolfsii* (Singh et al. 2013). Similar results were previously reported by Jain et al. (2012) where the consortium of beneficial microbes (*Trichoderma* spp., *Pseudomonas* spp., *Bacillus* spp.) were reported for the increment of various defense-related enzymes in pea plants against *S. sclerotinia* challenge. Another class of beneficial fungi *Piriformospora indica* has also been reported to cause elevation of defense response in plants against soil-borne pathogens (Serfling et al. 2007; Stein et al. 2008) showing its mechanism to be related with the upregulation of JA-mediated pathway.

A complex cross talk subsists between the plant and the beneficial microbes in response to the pathogenic microbes. The advancement in the research related to the responses triggered in plants against the pathogens by the beneficial microbes demonstrates the involvement of specific MAMPs analogous to the MAMPs of the pathogen highlighting the extensive coordination prevailing between different defense pathways involved.

Microbe-Mediated-Induced Lignification

Tertiary structure of lignin results from the polymerization of polyphenols and free radicals p-coniferyl, coumaryl, and sinapyl alcohols within the plant cell wall. This polymerization also results in the formation of covalent cross links with polysaccharide and protein moieties framing a tremendously resistant cell wall towards mechanical and enzymatic disruption against various classes of plant pathogens, insects, and herbivores (Bernards and Lewis 1998; Sederoff et al. 1999; Davin and Lewis 2000; Hatfield and Vermerris as 2001; Boerjan et al. 2003). Thus, lignin serves as physical defense shield in plant defense. Modifications in lignin composition, content, and distribution affect the strength of the shield which ultimately influences the agro-industrial pertinence of the plant material (Lewis and Yamamoto 1990).

Lignification obstructs phytopathogen growth on plant surface by a pentagonal approach (Ride 1978) as mentioned below:

1. Lignin deposition shields the plant tissue surface from enzymatic hydrolysis and mechanical penetration of phytopathogens by intensifying compressive forces between lignin layers preventing cellular penetration of phytopathogens.
2. Lignification of walls hinders the mobility of water and electrolytes between plant cells and phytopathogens facilitating pathogen killing by starvation.
3. Chemical modification of cell wall components is an effective strategy for disguising the pathogen enzymes. Coniferyl alcohol and ferulic acid covalently bound to cell wall glycoproteins and esterification of cell wall polysaccharides with cinnamic acid derivatives evidently reduces the cell wall damage

due to unavailability of substrates (Friend 1976; Whitmore 1978).

4. Generation of free radicals and low molecular weight phenolic precursors produced during polymerization of lignin may directly inactivate pathogens' membranes, enzymes, toxins, and elicitors.
5. Fungal walls contain chitin, cellulose, and hydroxyproline-rich proteins which can serve as matrices for lignin polymerization. Consequently, the hyphal tips become lignified and lose plasticity necessary for growth and penetration (Gottlieb and Pelczar 1951).

Among various mechanisms of plant defense, lignification is a strong structural defense strategy employed by plants to prevent pathogen penetration, and this relationship of lignification and disease resistance in plants is clearly witnessed in various studies. Comparatively, rapid lignin accumulation and deposition is observed in resistant cultivars than susceptible varieties (Vance et al. 1976; Yates et al. 1997; Durrant and Dong 2004). Not much is known about the role of rhizospheric microbes in strengthening plant's cell wall towards various biotic stresses. A recent study aimed to determine the efficacy of a triple microbial consortium of fluorescent *Pseudomonas* PHU094, *Trichoderma* THU0816, and *Rhizobium* RL091 strain on physiological defense responses in chickpea against the collar rot pathogen *S. rolfsii*. The result clearly illustrates the profound variation of lignin deposition in chickpea infected with *S. rolfsii*, which is attributed to different combinations of plant beneficial microbes to trigger lignification process. Interestingly, on treatment with triple consortium, uniform and maximum lignin deposition in the intrafascicular cambial cells was clearly observed and the phloem cells also displayed an enhanced lignification in sclerenchyma cap. Thus, claiming that beneficial rhizospheric microbes when employed in synergistic consortium can enhance the physical strength and durability of the cell wall towards cell wall-degrading phytopathogen (Singh et al. 2013).

In another study, alterations in phenolic metabolism and lignin deposition were analyzed in the roots of tomato plants after elicitation with *Fusarium* mycelium extract (FME), *Fusarium* culture filtrate (FCF), chitosan (CHT), and *Trichoderma* mycelium extract (TME). Maximum lignin synthesis was observed in plants treated with FME followed by CHT. Lignin deposition in the root cell walls increased to 5.7 times within 24 h after elicitation with FME. Similarly, CHT increased lignin deposition to almost five times, 24 h after elicitation. Thus, it was concluded that cell wall strengthening by lignin deposition was preceded by elicitation of lignin synthesizing enzymes revealing its essential role in defense response of tomato plants in response to various elicitors including one derived from *Fusarium oxysporum* f. sp. *lycopersici*, the causal organism of *Fusarium* wilt of tomato (Mandal and Mitra 2007).

Microbe-Mediated Quorum Sensing in Pathogen Management

The phenomenon of quorum sensing (QS) depicts the bacterial cell-cell communication and is generally cell density dependent. This cell-cell communication network is mediated by signal molecules (also called autoinducers), for example, oligopeptides and N-acylhomoserine lactones (AHL) in Gram-positive and Gram-negative bacteria, respectively. The QS plays a significant role in biofilm formation and in determining virulence factor in pathogenic bacterial species (Gram et al. 2002). In contrary, the phenomenon of antiquorum sensing (anti-QS) also exists where autoinducers interrupt with QS and thereby reducing the pathogenicity in several bacterial species (Truchado et al. 2012). Since QS contribute significantly in regulating virulence factor in many plant pathogens, anti-QS could be of great interest for decreasing pathogenic behavior and in developing innovative approach of disease control (Alvarez et al. 2012).

Considering the importance of anti-QS, presently, this phenomenon is getting noteworthy attention in plant disease management using bacterial biosensors and indicators. Interestingly, the cloning of *aiiA* gene (from *Bacillus* sp.) into transgenic potato and tobacco for enhancing

disease resistance against *Erwinia carotovora* is perhaps the very first example (Dong et al. 2001). Expression of this gene resulted in the production of AHL-lactonase in transgenic plants that interrupt with the QS systems arresting the virulence factor of *E. carotovora* leading to reduced disease incidence. Likewise, another example is the generation of transgenic tobacco lines using *expI* (*E. carotovora* AHL gene) which, after expression in plants, trap the pathogen in premature stage when it is unable to cause infection (Mäe et al. 2001). It is interesting to note that the autoinducer molecule AHL also contributes to the production of antimicrobial compounds in nonpathogenic *Pseudomonas chlororaphis* which has been successfully employed in suppressing plant diseases (Pierson et al. 1998a, b). Molina et al. (2003) evaluated the biocontrol potential of AHL-degrading *Bacillus* sp. A24 and genetically engineered AHL-degrading strain P3/pME6863 against soft rot in potato caused by *E. carotovora*. Recently, the novel approach of disrupting QS using structural analogs stimulating AHL-degradative microflora has been investigated by some researchers (Crépin et al. 2012a, b). In conclusion, these studies altogether clearly validate that the QS inhibition of phytopathogenic bacteria could be successfully employed for plant disease management. Hence, more focused researches are needed towards this approach.

Microbe-Mediated Nutrient Uptake and Defense

Fifty years back, the drive required for feeding the surplus population gave birth to the much talked about "Green Revolution" in India, leading to tremendous increase in food production. The unremitting use of fertilizers and pesticides has undoubtedly contributed to the increment in the food production but had led to the slow death of the soil microflora and fertility as well. In order to resolve the burning issue of deposition of toxic residues leading to biomagnifications in the food web, urgent need of using eco-friendly alternatives is required. Biopesticides comprising of microbial inoculants have emerged as a silver line for the current scenario with multifaceted benefits like safer approach both to the environment and to human kind, more targeted activity, low dose effectiveness, easily decomposable, natural propagation, and multiplication along with fortifying the plants' immune system (Berg 2009).

A constant conversation exists between the plants and the microbes in its vicinity. The plant-microbe interaction persists owing to the beneficial mutualism between the two partners which upshot various remuneration to plants as well as microbial commune. Positive effect on the growth and health of plants, enhanced stress tolerance, induction of disease reduction, biodiversity enrichment with ability to foster nutrient availability and uptake are the consequence of the interface between plant and microbes (Lugtenberg et al. 2002; Morrissey et al. 2004). These interactions are specific in terms of the host colonization due to the specific secondary metabolism and morphology (Berg and Smalla 2009). However, plant growth promotion and disease reduction or control have been the most noteworthy consequence of this interaction. Growth promotion in plants can be mediated through direct mechanisms by the microbes and also through indirect means through their antagonistic properties thereby reducing disease incidence allowing the healthy proliferation of the plants.

The competence of the microbes to colonize plant habitats is one of the crucial requirements for an effective plant-microbe interaction (Kamilova et al. 2005). Recognition, adherence, invasion (in case of endophytes and pathogens), colonization, and growth are the essential steps required for successful colonization apart from the various strategies employed by the microbial commune to establish the interaction. However, the initiation is executed by the plant itself by releasing signals recognized by the microbes which reciprocate the signals to initiate the colonization (Bais et al. 2006). Generally, motile organisms are preferred to participate and react in this cross talk (Lugtenberg et al. 2002).

Basically, three main types of interactions are related to the increment of plant growth mediated by beneficial microbes (Fig. 5.2). The most commonly known type of interaction is the

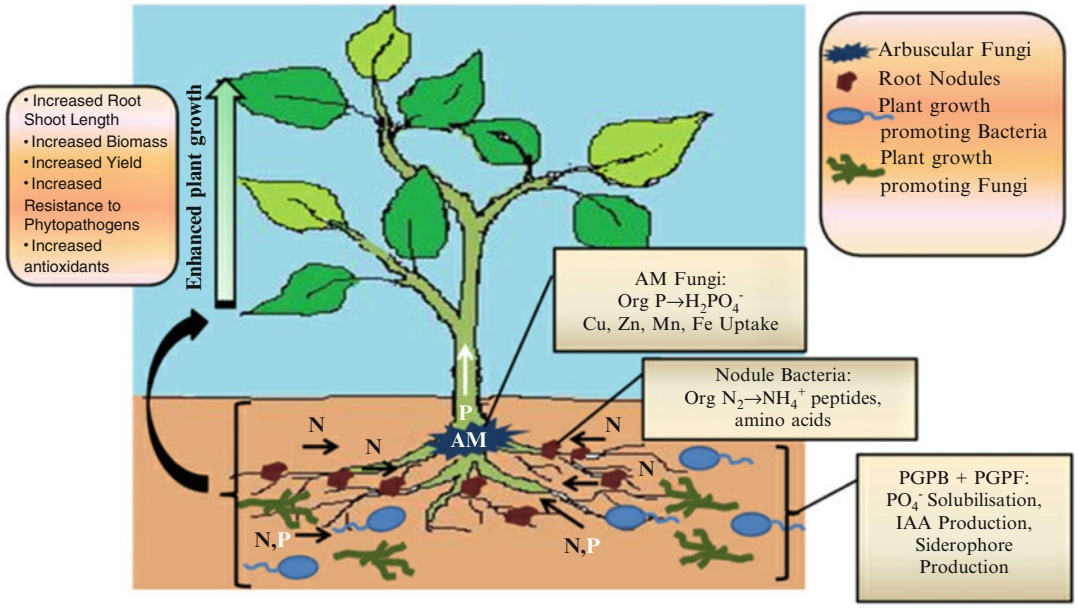

Fig. 5.2 An overview of microbe-mediated nutrient uptake in plants

symbiosis by *Rhizobium* species in fixing atmospheric nitrogen into ammonia in specific organs called nodules, found in leguminous plants (Van Rhijn and Vanderleyden 1995). Other important interaction is that of higher plants, commonly terrestrial flowering plants, with arbuscular mycorrhiza (AM) fungi which facilitate the absorption and translocation of phosphate from the soil (Harrison 1999). Lastly, the outcome of the numerous beneficial microbes that aid in mineralization of organic matter, thereby producing available nitrogen and phosphorous forms along with numerous micronutrients, provides the platform for the third type of interaction prevailing between plants and microbes (Hayatsu et al. 2008).

Phytohormones like indole-3-acetic acid (IAA), ET, cytokinins, and gibberellins are crucial for plant growth. Plants can obtain these hormones by either synthesizing themselves or by obtaining these from the microbes which can even alter the hormonal balance of the plant thereby causing alterations in the growth of the plants. Plant-associated bacteria have been shown to decrease the endogenous ACC levels thereby leading to increased root growth (Glick 2005). ACC deaminase-producing bacteria have also been reported to provide abiotic and biotic stress tolerance to plants thereby protecting them from the unfavorable conditions (Saleem et al. 2007). Seed treatment with auxin-producing *Pseudomonas fluorescens* has been reported to show stimulation of root growth, due to the production of nine times more tryptophan in root exudates of radish plants (Kamilova et al. 2006). This could be directly related to the growth promotion ability as the root growth-promoting hormone auxin is generally found in the root exudate which is synthesized from the amino acid tryptophan. Certain microbes like *B. subtilis*, *B. amyloliquefaciens*, and *Enterobacter cloacae* are known to enhance plant growth by releasing volatiles like 2, 3-butanediol and acetoin (Ryu et al. 2003). Increased photosynthetic efficiency and chlorophyll content were recorded in *A. thaliana* on treatment with *B. subtilis* GB03 which could be possibly related with the modulation of glucose and abscisic acid signaling (Zhang et al. 2008). Apart from auxin-producing bacteria, beneficial fungi like *Trichoderma* have been shown to be responsible for plant growth increment via auxin signaling (Contreras-Cornejo et al. 2009). Nitrogen fixation by the symbiotically associated *Rhizobium* species in the root nodules of leguminous plants or by free living bacteria like *Azospirillum*, *Burkholderia*, and *Stenotrophomonas* (Dobbelare et al. 2003) has been a major example

for nutrient acquisition to plants by microbes. Bacterial indirect contribution to plant growth by liberating phosphorous from organic compounds such as phytates play an important role in providing the necessary phosphorous required for proper growth of the plant (Unno et al. 2005). Another important nutrient, sulfate, is also made available to plants through oxidation by bacteria (Banerjee and Yesmin 2002). Siderophores have also been an important mode for the uptake of important micro-elements like Fe and other poorly soluble inorganic nutrients. Also, relation of siderophore production has been reported with the antagonistic activity against pathogens. *P. fluorescens* CHA0 has been reported to produce gluconic acid which acidifies the surrounding environment and thereby solubilizes the mineral phosphate in the soil creating a nutrient-limited condition for the plant pathogens (De Werra et al. 2009). Fungal biocontrol agent *Trichoderma* and mycorrhizal fungus *P. indica* have been reported to produce siderophore as a mechanism to check the growth of pathogens by creating a competitive environment for the availability of iron (Shoresh et al. 2010). Phosphate absorption to plants by AM has been well reported (Smith et al. 2011; Balakrishnan and Subramanian 2012). Also, increment in Cu, Zn, B, Mn, and Fe uptake in plants has been attributed to AM (Lambert et al. 1980; Clark et al. 1999; Liu et al. 2000).

Other important microbes that have been well studied for their mode of action and regulation in promoting plant growth and antagonism are members of the genera *Azospirillum* (Cassan and Garcia 2008), *Serratia* (De Vleeschauwer and Hofte 2007), *Stenotrophomonas* (Ryan et al. 2009), and *Streptomyces* (Schrey and Tarkka 2008) along with fungal genera *Ampelomyces*, *Coniothyrium*, and *Trichoderma* (Harman et al. 2004).

Microbe-Mediated Transgenerational Defense

Plant-recruited beneficial microbes can also prime the plants for enhanced defense responses, and the effect of priming could be passed on to the next generations as well. Priming of plants leads to enhanced perception of MAMPs, recognition of effector molecules secreted by pathogen, and recruitment of beneficial rhizospheric microbes (Conrath 2011). Recent understanding in the subject reflects that similar to animals, epigenetic inheritance in plants also takes place, and the epigenetic modifications of the chromatin as well as DNA methylation in plants could be well preserved in several subsequent generations (Pieterse 2012). Being sessile organisms, plants communicate with their offspring through this mechanism to "inform" the offsprings about the potential threats in their environment. Since, plants encounter potential biotic and abiotic threats from the environment at one or the other point of their life span, they are adapted to such mechanisms through the process of evolution for passing on the information to the next generation. Slaughter et al. (2012) demonstrated that the descendants of *A. thaliana* plants primed with an avirulent strain of *P. syringae* pv. *tomato* (PstavrRpt2) showed enhanced and rapid accumulation of defense-related gene transcripts associated with the SA signaling pathway. Further, the descendents also showed enhanced disease resistance against a virulent isolate of *P. syringae* and another oomycete pathogen *Hyaloperonospora arabidopsidis*. Interestingly, the progeny of transgenerationally primed plants when treated again with the priming agent displayed an even stronger primed phenotype. Recent evidences suggest that SA-mediated systemic resistance in plants also require chromatin remodeling and DNA methylation (Luna et al. 2012). Luna and Ton (2012) showed that transgenerationally acquired resistance was sustained through one stress-free generation, confirming epigenetic basis of the phenomenon. Failure of *non expressor of PR 1* (*npr1*) mutants to sustain transgenerationally acquired resistance in the progenies further signifies the SA-inducible pathway in this phenomenon and the central role of *NPR1*. Further, transgenerationally acquired systemic resistance was also demonstrated against biotrophic pathogens. A study on progenies obtained from diseased *Arabidopsis* also resulted in enhanced resistance towards the downy mildew pathogen (Luna and Ton 2012). Histone deacetylase 6 (HDA6) is a well-studied histone

deacetylase that has a prominent role in the silencing of genes. It was reported that HDA6 has also a significant role to play in the process of DNA methylation on its direct target locus. Thus, elucidation of the functions of HDA6 provided some very important clues of epigenetic regulation in plants (Kim et al. 2012). All these findings suggest the importance of transgenerationally acquired systemic resistance in plants and their potential role in managing plant diseases.

Future Prospects

The major challenge in the form of plant pathogens imposed to plant growth in natural and agricultural ecosystems urges for exploiting beneficial plant-microbe interactions. It is a well-known fact that microbial approach for plant disease management is necessary for maintaining the sustainability in agroecosystems. Therefore, it has been an emerging topic and gained considerable attention by many researchers. Though, various facets of plant-microbe interactions have long been studied, there is still a long way to go for achieving greater knowledge. The better understanding of plants' rhizosphere components is necessary to know the cross talk between plants and microbes. Linking this information to stress conditions would certainly provide a clue about developing a favorable and friendly environment for plant growth. Moreover, molecular approaches of studying the regulatory components of plant-microbe interactions can provide better understanding of improving such relationships by their manipulation.

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Rhizospheric Plant-Microbe Interactions: Key Factors to Soil Fertility and Plant Nutrition

6

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Abstract

Plant roots radiate a wide range of potentially valuable small molecular weight compounds into the rhizosphere which play a key role in the chemical, physical, and biological interaction between roots of the plants and the rhizosphere. The microorganisms present in the rhizosphere react with the numerous metabolites released by plant roots by positive, negative, and neutral ways, and these interactions may influence the plant growth and development, change nutrient dynamics, and also alter the plants susceptibility towards diseases and abiotic stresses. The root produces chemical signals that attract the bacteria and other microbes towards it. Beside this, positive interactions also include growth regulator mimics that support the plant growth and the cross-species signaling with other rhizospheric microorganisms. Plant-microbe interactions can influence the plant growth by providing nutrients and increased biotic and abiotic stress tolerance. Most of the agricultural soils have large amounts of inorganic and organic phosphorus (P), but it is present in immobilized form so is usually unavailable to plants. One of the major reasons why P is not readily available to plants is because of the high reactivity of P with some metal complexes. In this regard, the soil inoculants such as fungi, plant growth-promoting rhizobacteria (PGPR), and mycorrhizal fungi play a significant role in the solubilization of inorganic phosphate and mineralization of organic phosphates into easily available form to plants. Similarly, nitrogen (N) fixers provide available N to the plants. N is a key limiting factor in any ecosystem. For treating heavy metal-contaminated tailings and soils, bioremediation is one

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of the cost-effective methods and is emerging as the potential tool for removal of these contaminants from the soil or water. Bioremediation is a versatile process that could be applied in situ or ex situ manner. A wide variety of microorganisms such as bacteria, fungi, yeasts, and algae are being used in bioremediation processes, and some of these have already been employed as biosorbents of heavy metals. Various technologies such as phytoremediation, bioventing, bioleaching, land farming, bioreactor, composting, bioaugmentation, rhizofiltration, and biostimulation are nowadays used for the bioremediation of contaminants from the soil. The aim of this chapter is to focus on the plant-microbe interactions responsible for the maintenance of soil fertility, plant nutrition, and also the remediation of contaminated soil for sustainable agricultural system.

Introduction

Plants and their associated microorganisms play an important role in the formation or modification of soil (Pate and Verboom 2009). The soil formation takes place from the weathering of rocks and minerals and has distinct properties based on their origin, climate, and vegetation. Soil carbon is predominantly derived from plants, directly or indirectly, and while weathering may be due to physical and chemical influences. Most of the weathering processes involve primarily the plant roots, and the microbial activity may depend on the root-derived carbon (Beerling and Berner 2005). Next to water and temperature, nutrients are most crucial environmental factors desired for the development of the terrestrial plants. Rhizosphere is a zone of intense microbial activity, and the microorganisms present in the rhizosphere react with the various metabolites released by plant roots (Akhtar and Siddiqui 2010). Thus, the microorganisms and their products interact with plant roots in a variety of ways such as positive, negative, and neutral (Kuzyakov and Xu 2013). These interactions can influence the plant growth, change nutrient dynamics, and also alter the plants susceptibility towards the disease, abiotic stress, and resistance to heavy metals (Morgan et al. 2005).

The root systems support the aboveground part of the plant. In addition, the soil needs to maintain an appropriate pH, provide the protection from toxic substances and pathogens, and also contain suitable water levels. Besides this, all the essential mineral elements desired by plants are obtained from the soil. Marschner (1995) has recognized about 17 essential elements required for plant growth and reproduction. Amongst all the required essential elements, 14 elements are primarily acquired from the soil solution including the six macronutrients (N, P, K, Ca, Mg, and S) and 8 micronutrients (Fe, Cu, Zn, Mn, Mo, B, Cl, and Ni). Additionally, the plants also accumulate nonessential or toxic elements such as Cd, Pb, and Na from the soil solution.

Most of the essential elements are taken up in the ionic form from the soil solution by plants (White 2003). It is a well-known fact that the plant growth may be limited by the availability of essential elements or by the presence of toxic elements (Morgan et al. 2005). The interactions between microorganisms and the plant roots in rhizosphere assist the plants to acquire essential mineral nutrients and thwart the accumulation of toxic elements. Thus, it would be obvious that the structures of rhizospheric microbial communities are distinct from the bulk soil (Marilley and Aragno 1999), but vary in between plant species and over geographical time scale (Smalla et al. 2001). The different root zones in the same plant can hold distinct microbial communities reflecting qualitative and quantitative

differences in root exudation (Yang and Crowley 2000). Moreover, the structure of rhizospheric microbial communities could also be influenced by root infection by pathogenic microorganisms, which promote greater microbial community variability compared to healthy roots (Yang et al. 2001).

The Rhizosphere

Hiltner (1904) recognized rhizosphere as the volume of soil within immediate vicinity of the roots, which is predominantly affected by the activity of plants. The rhizosphere differs from the surrounding soils in most of the physicochemical and biological factors and with extensive microbial population both in numbers and diversity (Phillips et al. 2003). The number of microorganisms present in per gram of soil is much larger in the rhizosphere as compared to bulk soil. This increased microbial activity in the vicinity of the roots could be attributed to root exudates, sloughed senescent root cells, and mucigel described as rhizodeposition (Mukerji et al. 1998). Thus, the rhizosphere is the region in which the materials released from the root and root metabolic activities directly affect microbial density (Table 6.1). The roots continuously release volatile, soluble, and particulate materials through the process of rhizodeposition, and the growth of rhizospheric microorganisms on these materials turn over all the cellular activities and also release the nutrients in the form which could be utilized by plants.

The rhizosphere encompasses not only the region of nutrient uptake by the plant roots but also extends into the soil by the action of their byproducts (Van der Putten et al. 2001). This infusion of organic substrates into the rhizosphere by plants explains very clearly that the biomass and microbial activity are always greater in rhizosphere compared to bulk soil (Bardgett et al. 1998). Root tip is the site of root growth, usually characterized by rapidly dividing cells having the root exudates. The root exudates and sloughed root cells provide carbon to rhizospheric microorganisms, which in turn mobilize N and P in soil for the plants.

Table 6.1 Various root zones in the soil

Root zones	Functions
Rhizosphere	Region around the plant root where materials released from the root modify microbial populations and their activities
Endorhizosphere	Regions of the various cell layers of the root itself where microorganisms also colonize
Ectorhizosphere	An area surrounding the root and containing root hairs, plant, and bacterial mucilage
Rhizoplane	Root surface that can be colonized by microorganisms
Mycorrhizosphere	The ectorhizosphere extends a substantial distance from the root with the development of mycorrhizal fungal associations. Materials released from the fungus increase the microbial populations and their activities around the fungal hyphae
Spermosphere	The region around the germinating seed
Rhizodeposition	Release of materials from the roots

Chemical Compounds Produced by Plant Roots in the Rhizosphere

The general concept about the plant is that an aerial part such as stem and leaves contains greater biomass than roots, but this is actually a misleading impression. For many plants the biomass ratio of root and shoot is greater than the shoot and root biomass ratio. The materials released by plants include a wide variety of organic compounds (Table 6.2). The nature of these compounds depends upon various environment factors like temperature, moisture content, fertilizer dosages, herb, and plant age. The fine hairs of the roots are critical parts of the root system. They release various root exudate products into the environment due to their metabolic activity, and additionally a variety of gaseous metabolites also flow from the roots in the process of rhizodeposition.

Table 6.2 Different compounds released by plant roots in the process of rhizodeposition

Compounds	Exudates components
Amino compounds	Asparagine, α- alanine, glutamine, aspartic acid, leucine/isoleucine, serine, glycine, cystine/cysteine, methionine, phenylalanine, tyrosine, threonine, lysine, proline, tryptophan, β- alanine, arginine, homoserine, cystathionine
Fatty acids and sterols	Palmitic, stearic, oleic, linoleic, linolenic acids, cholesterol, campesterol, stigmasterol, sitosterol
Growth factors	Biotin, thiamine, niacin, pantothenate, choline, inositol, pyridoxine, N-methyl nicotinic acid
Nucleotides, flavonines, and enzymes	Flavonine, adenine, guanine, uridine/cytidine, phosphatase, invertase, amylase, protease, polygalacturonase
Organic acids	Tartaric, oxalic, citric, malic, propanoic, butyric, succinic, fumaric, glycolic, valeric, malonic
Sugars	Glucose, fructose, sucrose, maltose, galactose, rhamnose, ribose, xylose, arabinose, raffinose, oligosaccharide
Miscellaneous compounds	Auxins, scopoletin, fluorescent substances, hydrocyanic acid, glycosides, saponin (glucosides), organic phosphorus compounds, nematode-cyst or egg-hatching factors, nematodes attractants, fungal mycelium growth stimulants and inhibitors, zoospore attractants

Microbial Miscellanies in the Rhizosphere

The rhizosphere is the "cloud" of microbes surrounding the plant roots and is vital for the plant growth and survival. Plant roots construct novel environments for microbes due to change in increased levels of nutrients and intense microbial population (Giri et al. 2005). The microbial density is always high along the root away from the plant tip because respiration of the root is responsible for the change in the environment of the rhizosphere microbes. However, the microbial community developed in the changed environment will also face additional challenges like the availability of nutrients and edaphic and other biotic and abiotic factors which might be limiting for both the plant and their associated rhizospheric microbes.

The plant has an increasing demand for inorganic unavailable nutrients not present in a sufficient rate in the soil. In this regard, the rhizospheric microorganisms make a major contribution to overcome this demand (Table 6.3). The filamentous fungi also play a unique role in the nutrient uptake available to the plant due to their extensive hyphal network. They can derive the carbon from the plant and other limiting nutrients such as N and P from outside the root zone. Some free-living N_2-fixing bacteria like *Azotobacter*, *Azospirillum*, and *Azoarcus* present in the nitrogen-free or low nitrogen input environment play a significant role in the nitrogen fixation and also make the availability of nutrients to plant. Besides this, the rhizospheric community is not only enriched in bacterial and fungal populations but also has protozoans and nematodes. These patrons feed on the nutrient-rich bacteria and fungi and lead to more rapid turnover of the microbe populations in the rhizosphere which could be responsible for the acceleration in the release of nutrients for plant.

Plant-Microbe Interaction and Nutrient Availability

Continuous application of chemical fertilizers for enhancing soil fertility and crop productivity resulted in unforeseen harmful environmental effects such as leaching of nitrate into ground water, surface runoff of N and P, and eutrophication of aquatic ecosystems (Tilman 1998; Gyaneshwar et al. 2002). Besides this, it also negatively impacted the complex system of the biogeochemical cycles (Perrott et al. 1992; Steinshamn et al. 2004). All these events suggested that long-term application of chemical fertilizers not only reduced the soil fertility but also reduced the microbial population in the various agroclimatic conditions.

Despite the various negative effects on the environment, the use of total amount of fertilizers has increased globally to fulfill the food demand

Table 6.3 Microbial density of major groups of microorganisms present in the rhizospheric and non-rhizospheric soils

Microorganisms	Rhizosphere (microbes/g dry soil)	Non-rhizosphere (microbes/g dry soil)	R:S ratio
Algae	5×10^3	27×10^3	0.2
Actinomycetes	46×10^6	7×10^6	7.0
Bacteria	1200×10^6	53×10^6	23.0
Fungi	12×10^5	1×10^5	12.0

of the growing world population through intensive agriculture (Vitousek et al. 1997; Frink et al. 1999). In the last five decades, the rate of NPK fertilizer application has increased tremendously. Thus, there is a challenge to increase the agricultural productivity by minimizing the use of harmful chemical fertilizers. In this regard, the use of microbial inoculants such as PGPR and mycorrhizal fungi play a crucial role to minimize the demand of chemical fertilizers. Thus, the microbial inoculants are considered as promising components of agro-environmental integrated system because these inoculants possess the capacity to promote plant growth, enhance nutrient availability and uptake, and support plants' health (Barea et al. 1998; Dobbelaere et al. 2001; Hodge et al. 2001; Bonfante 2003; Vessey 2003; Kloepper et al. 2004; Han and Lee 2005; Adesemoye et al. 2008; Akhtar et al. 2011).

The arbuscular mycorrhizal (AM) fungi increase the plant growth by water and nutrient uptake (Ames et al. 1983; Akhtar and Siddiqui 2008a; Akhtar and Panwar 2011). The AM fungi have a high affinity towards P-uptake mechanism that enhances P nutrition in plants. The AM fungi could also scavenge the available P through their extraradical hyphae (Bianciotto and Bonfante 2002; Akhtar and Siddiqui 2008b; Akhtar et al. 2011). Apart from this beneficial association between the AM fungi and plant roots, there are few demerits as well. The AM fungi could not be cultured in vitro, due to their obligate nature, limiting the knowledge about the genetic basis of P solubilization and rhizosphere competence (Amijee et al. 1989; Koide 1991). Moreover, a high concentration of soil phosphate, above 100 ppm, could lead to the reduction in hyphal growth and chlamydospore production (Koide 1991). These limitations directly affect the P uptake and also cause the reduction of the benefits to plants (Stewart et al. 2005).

Similarly, the use of PGPR increases seed germination rate, root growth, yield, leaf area, chlorophyll content, nutrient uptake, protein content, hydraulic activity, tolerance to abiotic stress, and shoot and root weight, biocontrol and also delay the senescence (Raaijmakers et al. 1997; Bashan et al. 2004; Mantelin and Touraine 2004; Siddiqui et al. 2007; Bakker et al. 2007; Yang et al. 2009; Akhtar and Siddiqui, 2010). Other beneficial effects of PGPR include enhancing P availability (Rodriguez and Fraga 1999; Akhtar and Siddiqui 2008a; Akhtar and Panwar 2011; Yadav et al. 2012); fixing atmospheric nitrogen (Bashan et al. 2004; Gupta et al. 2012); sequestering iron for plants by production of siderophores (Raaijmakers et al. 1997; Bakker et al. 2007; Akhtar and Siddiqui, 2009), producing plant hormones such as gibberellins, cytokinins, and auxins (Gutierrez-Manero et al. 2001); and synthesizing 1-amino cyclopropane-1-carboxylate (ACC) deaminase (Glick et al. 2007a, b).

Availability of Nitrogen

Nitrogen is an essential element for plant growth and development, and the complex nitrogen cycle has a great impact on soil fertility (Jetten 2008). This cycle is conquered by four major steps, nitrogen fixation, nitrification, denitrification, and nitrogen mineralization (Ogunseitan 2005). It has been reported by earlier researchers that the microbial inoculants have significant roles in nitrogen cycling and utilization of nitrogenous fertilizers in the plant-soil system (Briones et al. 2003; Adesemoye et al. 2009) (Fig. 6.1). The uptake of nitrogen by leguminous (Elsheikh and Elzidany 1997; Akhtar and Siddiqui, 2006; Gupta et al. 2012) and nonlegume plants have been reviewed by many researchers (Kennedy et al. 1997; Dobbelaere et al. 2001;

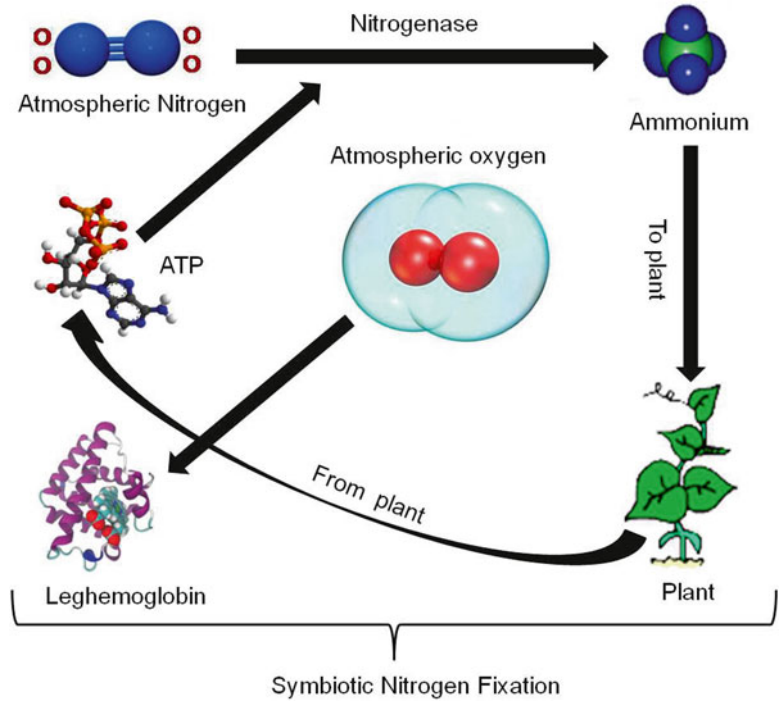

Fig. 6.1 Schematic representation of symbiotic nitrogen fixation in the leguminous plants

Vessey 2003; Egamberdiyeva and Hoflich 2004; Hernandez and Chailloux 2004; Wu et al. 2005; Shaharoona et al. 2008).

Wu et al. (2005) conducted greenhouse experiments on maize utilizing *Glomus mosseae* and *Glomus intraradices* with or without free-living nitrogen fixer, *Azotobacter chroococcum* and found that the co-inoculant increased the plant growth and NPK uptake and improved the soil properties in a much better way. Shaharoona et al. (2008) reported that inoculation of *Pseudomonas fluorescens* (strain ACC50) and *P. fluorescens* biotype F (strain ACC73) increased efficiency at all tested NPK fertilizer levels in wheat under pot and field trials. Amir et al. (2005) found that the inoculation of PGPR enhanced uptake of N and P in oil palm seedlings in nursery. Similarly, Aseri et al. (2008) conducted a field experiment to assess the effectiveness of PGPR (*A. chroococcum*) and AM fungi (*G. mosseae*) on the growth, nutrient uptake, and biomass production of pomegranate in individual or combined inoculations. The results showed that dual inoculation of PGPR and AM fungi led to higher biomass accumulation and uptake of N, P, K, Ca, and Mg. The result of the study thus confirmed that inoculation with mixed strains was more consistent than inoculation of single strain. It is well reported that the uptake of NPK and micronutrients are significantly enhanced in *Azospirillum* spp. inoculated plants under the greenhouse and field conditions. Thus, it would be very crucial to find out the factors behind the successful plant root colonization in *Azospirillum* and other PGPR which is responsible for the increase nutrient uptake.

Nitrogenase is the enzyme responsible for N_2-fixation. It has two components: I (a $\alpha 2\beta 2$ tetramer encoded by *nifD* and *nifK* genes) and II (a homodimer encoded by *nifH* gene). These two components are conserved in structure, function, and amino acid sequence throughout the diazotrophs. These genes are commonly reported to regulate lateral root development and long-distance movement of nitrogen (de Zamaroczy et al. 1989; Ueda et al. 1995; Minerdi et al. 2001). The nitrogenase enzyme complex has been credited for the capacity of PGPR to convert nitrogen into ammonia in a free state. Egener et al. (1999)

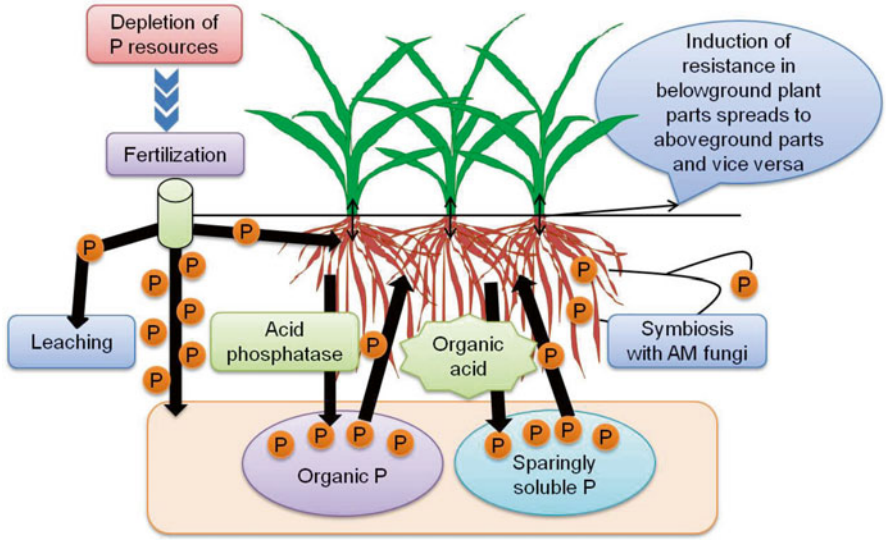

Fig. 6.2 Mechanism of soil P solubilization/mineralization and immobilization

studied root-associated GUS and *nifH* expression to monitor the establishment of N_2-fixing bacteria (*Azoarcus* sp.) on rice roots and successfully localized the expression of bacterial genes of interest in the host plant. They have found that the presence of combined nitrogen such as ammonia has a strong impact on the expression of *nif* gene in most diazotrophs. Similarly, Vande Broek et al. (1993) estimated the qualitative and quantitative associativeness in *nifH* expression in *A. brasilense* on wheat roots through *gusA* fusion plasmid system.

Availability of Phosphorus

Phosphorus is another growth-limiting nutrient generally present in the immobilized organic and inorganic form in the soil. Phosphorus is not readily available to plant due to its high reactivity with some metal complexes such as Fe, Al, and Ca leading to precipitation in the soil or may be present in very low concentration (usually in micromolar amount) (Igual et al. 2001; Gyaneshwar et al. 2002). It is also an important and well-known fact that when the P fertilizers are added to soils, they may not be absorbed or utilized by plants because of their sparingly soluble nature, and thus less amount of P would be available for the growth of agricultural crops (Gyaneshwar et al. 2002). Thus, the farmer may have to add large amount of fertilizers into the fields which later cause environmental problems (Ohno et al. 2005). The inoculants of PGPR and AM fungi play a significant role in the solubilization of inorganic phosphate and mineralization of organic phosphates (Mahmood et al. 2001; Tarafdar and Marschner 1994a; Tawaraya et al. 2006). Moreover, there are many evidences which are related to inorganic phosphate (Pi) transporter and its expression in the external hyphae of AM fungi, which is important in the uptake of P and transfer from the AM fungi to plants (Tarafdar and Marschner 1994b; Harrison and van Buuren 1995).

The mechanism for the solubilization of P was reported by Gharu and Tarafdar (2004) and Chen et al. (2006) (Fig. 6.2). The organic P usually accounting for 30–65 % of total P in soils must be converted to inorganic or low molecular weight organic acids before they could be assimilated by plants. The different forms of organic P in soils are inositol phosphatases, phosphoesters, phosphodiesters, and phosphotriesters. A large part of the organic P is present in the form of inositol

phosphatases (phytate) (Rodriguez and Fraga 1999; Zimmermann 2003). Phosphatases refer to any enzyme that can hydrolyze phosphate esters and anhydrides including phosphoprotein phosphatases, phosphodiesterases, diadenosine tetraphosphatases, exonucleases, 5′-nucleotidases phytases, phosphomonoesterases, alkaline, and acid phosphatases (Zimmermann 2003).

The role of phosphatases in the mobilization of phosphorus, originating from the organic soil sources, by AM fungi and PGPR has been reviewed by several researchers (Tarafdar and Marschner 1994b; Idriss et al. 2002; Rodriguez and Fraga 1999). Moreover, molecular tools have been also used to elucidate plant-microbe interactions in phosphorus metabolism (Rodriguez et al. 2000; Chen et al. 2006). Minder et al. (1998) indicated that the genetic control system of phosphate uptake is based on the phosphate regulatory protein *PhoB*, which is mediated by the transmembrane sensor protein *PhoR*. They have suggested that phosphorylated *PhoB* acts as a transcriptional activator to the *Pho* box in the promoter region of genes belonging to the *Pho* regulon and concluded that the product of the *PhoB* gene regulates the cellular response to environmental phosphate limitation. A study on *Bradyrhizobium japonicum* and soybean concluded that *PhoB* is responsible only for phosphate-limited growth, not for symbiotic nitrogen fixation (Minder et al. 1998). Subsequently, Ruiz-Lozano and Bonfante (1999) have investigated the role of *Burkholderia* sp. and AM fungi in P metabolism and found that through shunting off mechanism, the phosphorus is transferred from fungus to the plant. Ruiz-Lozano and Bonfante (1999) also characterized an operon *Pst*-like system in *Burkholderia* similar to *E. coli* phosphorus uptake by *Gigaspora margarita*. By the possession of a DNA region with nitrogenase-coding genes (*nif* operon), the *Burkholderia* sp. could also affect nitrogen uptake. The approaches are promising to elaborate the role of the interaction of bacteria and AM fungi in the nutrient uptake (Akhtar 2011).

Availability of Other Nutrients

Microbial inoculants have shown their influence towards the uptake of other nutrients besides N and P (Peix et al. 2001; Khan 2005; Wu et al. 2005; Adesemoye et al. 2008). Khan (2005) observed that inoculation of *Pseudomonas* and *Acinetobacter* strains resulted in enhanced uptake of Fe, Zn, Mg, Ca, and K by crop plants. In another study, inoculation of chickpea and barley with strains of *Mesorhizobium mediterraneum* significantly increased the K, Ca, and Mg in addition to P and N in both crop plants (Peix et al. 2001). Kohler et al. (2008) have demonstrated the effects of PGPR (*Pseudomonas mendocina*) and AM fungi (*G. intraradices* and *G. mosseae*) on uptake of N, P, Fe, Ca, and Mn in lettuce under three different levels of water stress. Sheng and He (2006) reported improved uptake of K by the inoculation of *Bacillus edaphicus* and suggested that the production of organic acids (citric, oxalic, tartaric, succinic, and α-ketogluconic) by this strain leads to chelation of metals and mobilization of K from K-containing minerals. Similarly, Giri and Mukerji (2004) reported a significant increase in Mg concentrations in the seedling of *Sesbania aegyptiaca* and *Sesbania grandiflora* by the application of *Glomus macrocarpum*, compared to non-mycorrhizal seedlings in saline soil. Liu et al. (2000) reported an increase in acquisition of Fe, Zn, Cu, and Mn by mycorrhizal fungi in maize. Moreover, sulfur uptake has been achieved through sulfur oxidization (Banerjee et al. 2006) and iron uptake through siderophore-producing bacteria (Bakker et al. 2007). Biswas et al. (2000) reported a significant increase in Fe uptake in lowland rice through inoculation of *Rhizobium leguminosarum* bv. *trifolii* and suggested that the increase in uptake of Fe, P, and K was associated with higher nitrogen rates.

Plant Interactions for Remediation of Contaminated Soils

Soil is an important habitat for thousands of organisms including a variety of fungi, actinobacteria, algae, protozoa, and different types of bacteria. These microorganisms in association with soil particles or soil organic matter in the rhizosphere are essential for the plant. Plant-microbe interactions are now being intensively investigated for decontamination and remediation

processes. With the discovery of a number of soil microorganisms that are capable of degrading xenobiotic chemicals including herbicides, pesticides, solvents, and other organic compounds, microbial degradation might provide a reasonable and effective means of disposing toxic chemical wastes. Due to the sensitivity and the sequestration ability of the microbial communities towards the heavy metals, microbes have been used for bioremediation of sites contaminated with them (Hallberg and Johnson 2005; Kao et al. 2006; Umrania 2006). Although microbial communities in metal-polluted bulk soils have been studied, there are a limited number of studies on the composition of microbial community in the plant rhizosphere growing in soils highly polluted with heavy metals (Dell'Amico et al. 2005).

Phytoremediation

Phytoremediation is a kind of bioremediation technique that uses plants to manage or remediate polluted soils. It is an emerging and cost-effective technology. It could be defined as "the elimination, attenuation, or transformation of polluting or contaminating substances by plants into their less toxic forms" (Vidali 2001; Kavamura and Esposito 2008). It can be used as in situ or ex situ technology. Soils could be contaminated with thousands of contaminants varying in their composition and concentration through inadequate residue disposal, accidental wastes, and inappropriate use (Knaebel et al. 1994). These contaminants include nitrates, phosphates, and perchlorates (Nozawa-Inoue et al. 2005); explosives such as hexahydro-1,3,5-trinitro-1,3,5-triazine and octahydro-1,3,5,7-tetranitro-1,3,5,7-tetrazocine (Kitts et al. 1994); monoaromatic hydrocarbons like benzene, toluene, ethylbenzene, and xylene (Rooney-Varga et al. 1999); polycyclic aromatic hydrocarbons (Wang et al. 1990); herbicides such as diuron, linuron, and chlorotoluron (Fantroussi et al. 1999); and heavy metals (Glick 2003).

In case of soil remediation, several factors such as soil characteristics, type, and concentration of contaminants should be considered (Boopathy 2000; Sheoran et al. 2008). The remediation of the harmful contaminants from the soil by plants is an emerging alternative to restore the contaminated sites (Singh et al. 2003; Paquin et al. 2004; Vassilev et al. 2004; Shah and Nongkynrih 2007; Padmavathiamma and Li 2007; Rajkumar and Freitas 2008; Lone et al. 2008; Akhtar et al. 2013). Phytoremediation can be classified according to the method and the nature of contaminants (Lasat 2002; Eapen et al. 2003; Newman and Reynolds 2004; January et al. 2008). The various methods used for the phytoremediation are discussed below (Fig. 6.3).

Phytoextraction

Plants can absorb the concentrated metals in their aboveground parts which can then be harvested. Brennan and Shelley (1999) found that plants have the capability to extract large concentrations of heavy metals into their roots, translocate them to the stem, and produce a large quantity of plant biomass.

Phytodegradation

It is also known as phytotransformation. In this process, plants degrade organic pollutants directly via their enzymatic activities. Some enzymes break down and convert ammunition wastes, others degrade chlorinated solvents such as trichloroethylene, and others degrade herbicides.

Phytovolatilization

Phytovolatilization refers to the uptake and transpiration of contaminants, primarily organic compounds, by plants. The contaminant, present in the soil solution, is taken up and modified by the plant and is released to the atmosphere through the plant leaves by evaporation or vaporization processes.

Phytostimulation

It refers to stimulation of rhizospheric microorganisms capable of degrading the contaminants by the growing roots releasing exudates/nutrients such as carbon sources. This method is useful in removing organic contaminants, such as pesticides, aromatics, and polynuclear aromatic hydrocarbons from soil and sediments (Ukiwe et al. 2013).

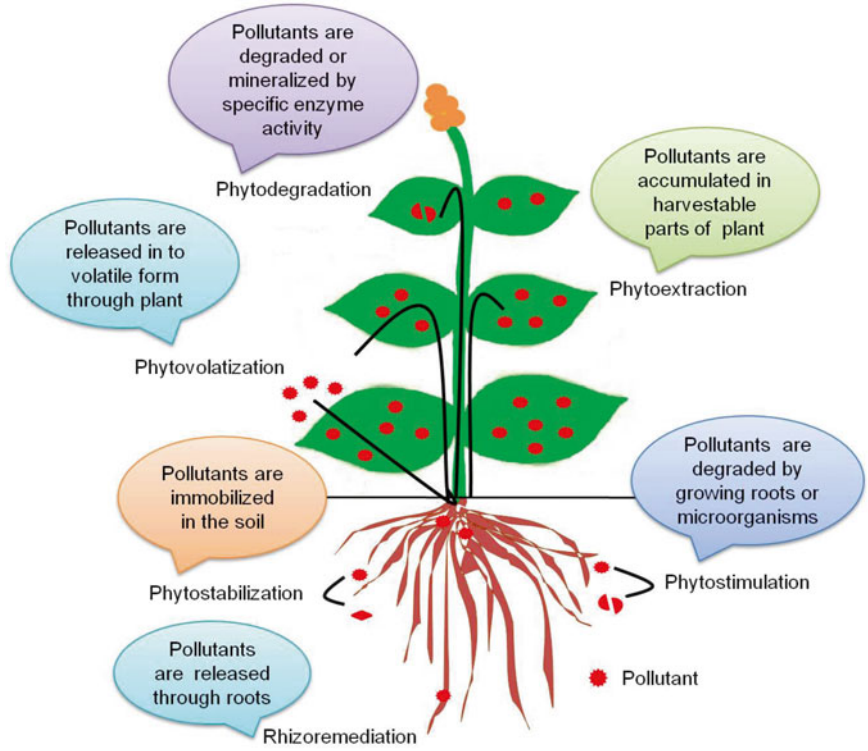
Pollutants are degraded or mineralized by specific enzyme activity
Phytodegradation
Pollutants are accumulated in harvestable parts of plant
Phytoextraction
Pollutants are released in to volatile form through plant
Phytovolatization
Pollutants are immobilized in the soil
Phytostabilization
Pollutants are degraded by growing roots or microorganisms
Phytostimulation
Pollutants are released through roots
Rhizoremediation
Pollutant

Fig. 6.3 Schematic diagram of different approaches of phytoremediation

Phytostabilization

In this method, the use of plant roots may limit the contaminant by reducing its mobility or leaching in the soil. Plants decrease the amount of water percolating through the soil matrix, which may act as a barrier and reduce the leaching of the contaminant. Phytostabilization can occur through sorption, precipitation, complexation, or metal valence reduction. It is helpful in the treatment of contaminated land areas affected by mining activities (Raskin and Ensley 2000). Phytostabilization is commonly used to treat the metal (arsenic, cadmium, chromium, copper, and zinc) contaminants (Kunito et al. 2001).

Rhizofiltration

It could be used for metals such as Pb, Cd, Cu, Ni, Zn, and Cr which are retained within the roots. It is useful for both terrestrial and aquatic plants for in situ or ex situ purposes. In this method, the contaminants do not translocate to the shoots. The terrestrial plants are more favored for rhizofiltration due to their fibrous and elongated root systems. The main limitation of this method is to adjust to the pH at regular intervals.

Rhizoremediation

In this method, microorganisms are utilized in combination with the plants (Jing et al. 2007). Generally organic pollutants with high hydrophobicity (hence, unable to be absorbed by the plant) are remediated by this method. Microbes play major role in this method. Plants mainly provide the microbes with nutrients and growth factors to proliferate (Siciliano and Germida 1998; Chaudhry et al. 2005).

Rhizoremediation of Organic Contaminants by PGPR

Initially, the PGPR were used for plant growth promotion and biocontrol of plant diseases, but now they are also being used for rhizoremediation of

organic contaminants (Narasimhan et al. 2003; Huang et al. 2004, 2005). In contrast to inorganic compounds, microorganisms can degrade and mineralize organic compounds in association with plants (Saleh et al. 2004). Brazil et al. (1995) reported that the bacteria are capable of degrading a certain kind of organic pollutants such as polychlorinated biphenyls isolated from various locations and studied the encoding genes involved in this pathway. Rhizoremediation of various organic pollutants is known when a particular crop plant in combination with their known or unknown microbes are used as reviewed in detail by Kuiper et al. (2004).

Rhizoremediation of Metals by PGPR

A wide range of plants have been tested for their ability to take up high levels of metals by roots form soil and translocate these metals into the leaves and shoots. The use of PGPR as adjuncts in metal phytoremediation can significantly facilitate the growth of plants in the presence of high levels of metals (Zhuang et al. 2007; Glick 2010).

Rhizoremediation by Endophytic Microorganisms

Endophytic bacteria could be defined as bacteria colonizing the internal tissues of plants without causing infection or negative effects on their host (Lodewyckx et al. 2002). With the exception of seed endophytes, the primary site where endophytes gain entry into plants is via the roots (Pan et al. 1997; Germaine et al. 2004). The endophytes either reside inside the plants in specific plant tissues like root cortex and xylem or colonize the plant systematically by transport through the vascular system or apoplast (Mahaffee et al. 1997; Quadt-Hallmann et al. 1997). Endophytic bacteria have been isolated from a variety of healthy plant species ranging from herbaceous crop plants (Lodewyckx et al. 2002; Malinowski et al. 2004; Mastretta et al. 2009), different grass species (Zinniel et al. 2002; Dalton et al. 2004), to woody tree species (Cankar et al. 2005; Moore et al. 2006; Taghavi et al. 2009).

Pseudomonas, *Burkholderia*, and *Enterobacter* are amongst the most common genera of cultivable endophytes (Mastretta et al. 2006). In addition to their beneficial effects on plant growth, endophytes have also been used in phytoremediation (Weyens et al. 2009). Idris et al. (2004) investigated the endophytes from *Thlaspi goesingense*, a hyperaccumulator of Ni in both cultivation-dependent and cultivation-independent techniques. They concluded that the endophytes used in the cultivation-independent techniques have the potential to tolerate higher concentration of Ni than rhizospheric bacteria. This kind of technique is very promising in the phytoremediation of heavy metals, but the actual mechanisms is not well understood, and its application in the phytoremediation of heavy metal is expensive and very complicated (Weyens et al. 2009).

Mycorrhizoremediation

In this advanced approach, symbiotic AM fungi could be used for phytoremediation (Huang et al. 2004; Khan 2006). AM fungi have the potential to efficiently explore the soil volume (Meharg and Cairney 2000). Mycorrhizal association exhibits substantial resistance against the toxic metals (Leyval et al. 1997; Meharg and Cairney 2000) and organic compounds such as m-toluate and petroleum polycyclic aromatic hydrocarbons (Sarand et al. 1998, 1999; Leyval and Binet 1998).

In addition to their protective behavior, mycorrhizae may contribute to resistance of plant-microbial associations through enhanced degradation of organic pollutants in the mycorrhizosphere and lowering the bioavailable concentration of heavy metals in soil (Meharg and Cairney 2000). It is evident from the reports of the earlier researchers that the AM fungi have the potential to increase the uptake of various heavy metals in plants (Liao et al. 2003; Whitfield et al. 2004; Liu et al. 2005; Leung et al. 2006). However, some other studies showed there is no effect of AM fungi or even decreased concentrations in plant tissues (Trotta et al. 2006; Wu et al. 2007). On the basis of contrasting results, it is very difficult to evaluate the potential of mycorrhizal fungi in the uptake of heavy metals in field experiments (Liu et al. 2005; Leung et al. 2006; Wu et al. 2007; Wenzel 2009).

Bioremediation by Microbes

In this process, microorganisms are used for degradation or removal of contaminants from the soil. This method of degradation or removal of environmental contaminant through microbial activity is cost-effective and environmentally safe compared to other physicochemical methodologies used for bioremediation (Akhtar et al. 2013). It could be used both as in situ and ex situ methods. However, there are many loopholes and black holes in this technology such as the use of inappropriate microbial system for removal of metal toxicity, low or inactive microbial populations, or presence of complex pollutant mixtures. The rate and extent of biodegradation depend upon many factors which have been summarized in tabular form (Table 6.4).

For endurance under metal-stressed environment, PGPR have evolved several mechanisms by which they can immobilize, mobilize, or transform metals rendering them inactive so as to tolerate the heavy metal ions. These mechanisms include: (1) exclusion, the metal ions are kept away from the target sites; (2) extrusion, the metals are pushed out of the cell through chromosomal/plasmid mediated events; (3) accommodation, metals form complex with the metal binding proteins or other cell components; (4) bio-transformation, toxic metal is reduced to less toxic forms; and (5) methylation and demethylation. Thus, in general, the immobilization and mobilization are the two main techniques used for the bioremediation of metals by microbes.

Table 6.4 Some major factors affecting the bioremediation process

Factors	Affect
Microbial	Growth until critical biomass is reached, mutation and horizontal gene transfer, enzyme induction, enrichment of the capable microbial populations, and production of toxic metabolites
Environmental	Depletion of preferential substrates, lack of nutrients, and inhibitory environmental conditions
Substrate	Too low concentration of contaminants, chemical structure of contaminants, toxicity of contaminants, and solubility of contaminants
Biological aerobic vs. anaerobic process	Oxidation/reduction potential, availability of electron acceptors, and microbial population present in the site
Growth substrate vs. co-metabolism	Type of contaminants, concentration, alternate carbon source present, and microbial interaction such as competition, succession, and predation
Physicochemical bioavailability of pollutants	Equilibrium sorption, irreversible sorption, and incorporation into humic matters
Mass transfer limitations	Oxygen diffusion and solubility, diffusion of nutrients, and solubility and miscibility in water

Immobilization Techniques

Immobilization is a technique used to reduce the mobility of contaminants by altering the physical or chemical characteristics of the contaminant. This remediation approach can utilize microorganisms to immobilize metal contaminants. It is usually accomplished by physically restricting contact between the contaminant or by chemically altering the contaminant (Evanko and Dzombak 1997, Mulligan et al. 2001; Akhtar et al. 2013). Chemical reagents and bacterial reagents assist with the immobilization of metal contaminants. Most sites contaminated with metals use the solidification and stabilization approach to immobilize metals. Solidification treatment involves mixing or injecting chemical agents to the contaminated soil. The prominent mechanism by which metals are immobilized is by precipitation of hydroxides. The chemical composition of the site, the amount of water present, and the temperatures are all factors important to the successful use of the solidification/stabilization mechanisms (Evanko and Dzombak 1997; Wuana and Okieimen 2011). The stabilization and solidification technique is achieved by mixing the contaminated material with appropriate amounts of stabilizer material and water. The mixture forms a solidified matrix with the waste. The stabilization and solidification techniques can occur both in situ and ex situ. In situ is

preferred for volatile or semi-volatile organics. The in situ process is useful for treating surface or shallow contamination.

Mobilization

Microorganisms can mobilize metals through autotrophic and heterotrophic leaching, chelation by microbial metabolites and siderophores, methylation, and redox transformations. Heterotrophic leaching is when microorganisms can acidify their environment by proton efflux thus leading to the acidification resulting in the release of free metal cations. Autotrophic leaching is when acidophilic bacteria retrieve CO_2 and obtain energy from the oxidation of the ferrous iron or reduced sulfate compounds, which causes solubilization of metals (Agrawal 2005). Siderophores are specific iron-chelating ligands and are able to bind to other metals, such as magnesium, manganese, chromium, and gallium, and radionuclide, such as plutonium (Akhtar et al. 2013). Methylation involves methyl groups that are enzymatically transferred to a metal, forming a number of different metalloids. Redox transformations can allow microorganisms to mobilize metals, metalloids, and organometallic compounds by reduction and oxidation processes. There are various metal-mobilization techniques that can also occur in nature (Gadd 2004).

Conclusion

The rhizospheric microorganisms can influence plant growth, nutrition availability, disease susceptibility, resistance towards heavy metals, and various abiotic stresses. Plant growth attributes could be limited by the unavailability of essential elements or the presence of toxic elements. The interactions between plant roots and microorganisms present in the rhizosphere assist them to acquire essential mineral nutrients from the soil and prevent the accumulation of toxic elements. Amongst various rhizospheric microorganisms, free-living and symbiotic nitrogen-fixing bacteria contribute a lot to meet this demand. In the presence of N-free or low nitrogen content, the rhizospheric bacteria accomplish associative nitrogen fixation and thus provide essential nutrients to plants. Moreover, the phosphate-solubilizing bacteria have the capacity to convert inorganic unavailable P form to soluble forms available to plants.

Of the various microorganisms present in the soil, the rhizospheric bacterial community has the potential to increase the plant growth and minimize the disease severity and also is useful in the degradation or removal of toxic elements from water, soil, sludge, and process-waste stream through bioremediation. These technologies could be broadly classified as ex situ and in situ. The ex situ technologies are applied for the physical removal of the contaminated materials for treatment process, while in situ techniques for the treatment of contaminated materials in place. Plant-microbe interactions can thus be applied for diverse aspects by the development of sustainable technologies for enhancement of crop yield, suppression of phytopathogens, degradation of pollutants, and remediation of contaminated sites.

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Plant-Endophyte Interaction and Its Unrelenting Contribution Towards Plant Health

7

Sarangthem Indira Devi and P. Momota

Abstract

Microbes are ubiquitous in nature; likewise, they form a part of flora in the plant species either actively colonizing or latently dormant. Beneficial plant-microbe interactions that promote plant health and development in a broad spectrum of area have been a subject of substantial study. Beneficial microbial associations like mutualists and symbiotic associates in plants show considerable promise due to the ability to provide ample benefits not only to the host plant but also a great prospect to mankind. Endophytes represent a hot spot area of current research, considering their 360 degrees of contribution and a wide range of applications in agricultural science, as potent plant growth promoters and emerging as effective biocontrol agents against various severe plant pathogens, limiting the use of chemical pesticides and other hazardous chemicals. Endophytes also share their contribution to modern medicine, industrial perspectives, nanoscience, forest management, and bioremediation. The endophytes may produce a plethora of substances or compounds with potential therapeutic applications covering plants to humans. There is increase in studies of endophytes for improving yield of food crops and the sustainable production of nonfood crops for biomass and biofuel production. The ability to tolerate both biotic and abiotic stresses is also an added advantage over the other competent microbial flora of its surrounding habitat. Indeed, endophytes are a golden area of researching and exploiting the potent functional properties that aid in understanding the depth of plant-microbe interactions and their contribution to sustainable agriculture, being the focus of concern in the present food crisis scenario.

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Introduction

Research on the bacteria residing in the internal tissues of plants began in the 1870s by Pasteur and others (Hollis 1949). The German botanist, Anton de Bary, known as the father of plant pathology, coined the term endophyte in 1886 giving a description to microorganisms that colonize the internal tissues of stems and leaves (Wilson 1995). The presence of endophytes was first recorded by Vogl in 1898 revealing a mycelium residing in the grass seed of *Lolium temulentum* (Wilson 1996). Later on the term endophyte was used to denote those microorganisms that colonize the plant tissues without causing any symptom throughout their existence in the host. Defining endophyte is simple yet complex, and the term updates with inputs on the current findings; however, endophyte is named by the location and not just with the relationship or interaction it shares with the infected host or the numerous functions it imparts, benefitting the host plant. Microorganisms that gain access to the internal tissues of a plant are termed endophytes, but true endophytes are the ones that colonize internally without causing any disease symptoms throughout their short to long life span in the host. Endophytes with the potential to cause symptoms could be categorized under groups linking to opportunistic, latent pathogens and latent saprophytes. The potential importance of endophytic fungi became clear in 1975, when Charles Bacon discovered endophytes of pasture grasses in the family *Clavicipitaceae* which were toxic to cattle (Bacon et al. 1997). Beneficial endophytic microorganisms comprise especially of fungi and bacteria that colonize the internal plant tissues without causing damage to their host (Petrini 1991).

Endophytes are categorized into three functional groups mainly, plant growth promoters, biocontrol (Bashan and Holguin 1998), and plant stress homeo-regulating microorganisms (Cassan et al. 2009) that can directly or indirectly facilitate the plant growth in optimal, biotic, or abiotic stress conditions. The colonization of plant tissues by endophytes involves several steps including host recognition, spore germination, penetration of the epidermis, and tissue colonization (Petrini 1991, 1996). Studies on microorganisms from plant species are recently becoming more frequent since these microorganisms have widely been explored and investigated for mechanism of biological control, production of novel metabolites with pharmacological properties/importance, and as a potential source for bioremediation. Endophytes intrude phytopathogens and hence have been used as biocontrol agents against microbial plant pathogens, insects, nematodes, and plant pests. Endophytes can promote overall plant growth under biotic (pathogenic microbes, insects, nematode) and abiotic (drought, temperature, pH, salinity, flood, wound, cut or injured part, nutrient deprived) stress conditions. Attributes have been credited to their ability to produce novel bioactive compounds in order to survive in the host environment. One of the considerable roles of endophytic microbes is to initiate the biological degradation of dead or dying host plant, which is necessary for nutrient cycling (Strobel et al. 2002).

Further, endophytic fungi may also produce metabolites with a thermoprotective role, e.g., plants of some volcanic areas in the USA were found to be colonized by the endophytic fungus *Curvularia* species. Plants grown from surface-sterilized seeds in sterile soil that had been incubated with the organism survived constant soil temperature of 50 °C, while the non-symbiotic plants died (Redman et al. 2002). The study of endophytes is expected to become an important component of microbial biology in the future. In forest ecosystems, endophytes contribute by effectively increasing phenotypic plasticity of their long-lived tree hosts under variable or deleterious environmental conditions. Various nanoparticles were reported to be synthesized by a countable number of microorganisms including endophytes. Recently, endophytic actinomycetes have been recognized as potential organisms for biosynthesis of nanoparticles like gold and silver. Nanoparticles from an endophyte may again be used for curing dreaded diseases in the near future with advancement in endophyte research. Therefore, endophytes will play a great role in the future with production and knowledge of

more efficient, productive, and cost-effective compounds including nanomedicine for the disease control of humans, animals, and plants. To date, only a few plants have been extensively investigated for their endophytic biodiversity and their potential to produce bioactive secondary metabolites. Various culture-dependent and culture-independent approaches have been adopted to gain maximum possible endophytic isolates. Culture-independent approaches focus on molecular techniques such as quantitative polymerase chain reaction in real time, denaturing gradient gel electrophoresis (DGGE), multivariate analysis to microbial ecology data, clone library construction analysis, and the upcoming high-throughput methodologies such as microarrays and pyrosequencing. Molecular approaches for the isolation and characterization of bacterial endophytes and plant-associated bacterial communities have been reviewed recently by Franks et al. (2006).

The relationship of an endophyte with single or multiple plant hosts can be described in terms of host specificity, host recurrence, and host selectivity or host preference (Zhou and Hyde 2001; Cohen 2006). Consequently the opportunity to find new endophytic microorganisms with potential beneficial properties among a diversity of plants in different ecosystems is intriguing and can lead to identification of novel compounds for drug discovery and developmental strategies in the treatment of catastrophic diseases in humans, animals, and plants.

Ecological Colonization and Biodiversity

Endophytes are sheltered from environmental stress and microbial competition by the host plant, and they seem to be ubiquitous in plant tissues, having been isolated from various parts of plants like flowers, fruits, leaves, stem, root, and seeds in various plant species (Kobayashi and Palumbo 2000). Plants growing in areas of great biodiversity usually have the prospect of harboring endophytes with great biodiversity (Strobel and Daisy 2003). The mode of colonization is systemic with bacterial colonies and biofilms residing latently in intercellular spaces, inside the vascular tissue or within cells (Ulrich et al. 2008). It seems obvious that endophytes are rich and reliable sources of genetic diversity and novel undescribed species. Endophytic fungi that live inside the tissue of living plants are an underexplored group of microorganisms. Dreyfuss and Chapela (1994) estimated that there may be at least one million species of endophytic fungi alone! Specifically, the bacteria enter tissues via germinating radicals, secondary roots, or stomata or as a result of foliar damage. An important aspect of colonization is the ability to compete with indigenous microorganisms already present in the soil and rhizosphere of the developing plant. Our understanding of the factors involved in these interactions has been hindered by our inability to culture and characterize diverse members of the rhizosphere community and to determine how that community varies with plant species, plant age, location on the root, and soil properties. Molecular studies on endophytes have revealed a large richness of species. Endophytic bacterial diversity was estimated in Mexican husk tomato plant roots by amplified rDNA restriction analysis and sequence homology comparison of the 16S rDNA genes. The predominant genera were *Stenotrophomonas* (21.9 %), *Microbacterium* (17.1 %), *Burkholderia* (14.3 %), *Bacillus* (14.3 %), and *Pseudomonas* (10.5 %). The endophytic bacterial diversity within the roots of Mexican husk tomato plants is a subset of the rhizosphere bacterial population, dominated by a few genera (Marquez-Santa Cruz et al. 2010).

The diversity of cultivable bacterial endophyte is exhibited not only in the variety of plant species colonized but also in the many taxa involved, with most being members of common soil bacterial genera such as *Enterobacter*, *Pseudomonas*, *Burkholderia*, *Bacillus*, and *Azospirillum* (Lodewyckx et al. 2002). A recent study (Magnani et al. 2010) describes the diversity of endophytes found in Brazilian sugarcane, reporting the endophytic bacteria in sugarcane to be more diverse than previously reported and also demonstrate niche specificity. Tropical and temperate

rain forests are the most biologically diverse terrestrial ecosystems on Earth. The most threatened of these spots cover only 1.44 % of the land's surface, yet they harbor more than 60 % of the world's terrestrial biodiversity (Mittermeier et al. 1999). Five taxa exhibiting the most promising levels of colonization and ability to persist were identified (by 16S rRNA gene sequence, fatty acid and carbon source utilization analyses) as *Cellulomonas*, *Clavibacter*, *Curtobacterium*, *Pseudomonas*, and *Microbacterium* (Elvira-Recuenco and Van Vuurde 2000).

Autofluorescent protein (AFP) methods are now a key tool for studying processes such as microbe-plant interactions and biofilm formation (Larrainzar et al. 2005). These techniques have been utilized to detect and enumerate microorganisms in situ on plant surfaces and *in planta* (Tombolini and Jansson 1998). AFP strategies use a marker system, which encodes the green fluorescent protein (GFP). GFP is a useful AFP biomarker because it does not require any substrate or cofactor in order to fluoresce. Endophyte colonization has also been visualized with the use of the b-glucuronidase (GUS) reporter system. A GUS-marked strain of *Herbaspirillum seropedicae* Z67 was inoculated onto rice seedlings. GUS staining was most intense on coleoptiles, lateral roots, and also at some of the junctions of the main and lateral roots (James et al. 2005). The study showed that endophytes entered the roots through cracks at the point of lateral root emergence. *H. seropedicae* subsequently colonized the root intercellular spaces, aerenchyma, and cortical cells, with a few penetrating the stele to enter the vascular tissue. The xylem vessels in the leaves and stems were also colonized. Successful endophyte colonization also involves a compatible host plant. Recent investigations focus on the obligate nitrogen-fixing endophyte *Azoarcus* sp. strain BH72, which expresses nitrogenase (nif) genes inside rice roots (Miche et al. 2006). They used a proteomic approach to dissect responses of rice roots toward bacterial colonization and jasmonic acid treatment (which induces plant defense proteins). Data suggests that induced plant defense responses may contribute to restricting endophytic colonization in grasses.

Plant-Endophyte Interaction and Its Genomic Review

Genome analysis of currently sequenced endophytic microbes demonstrates that diverse genome characteristics and sets of host interaction with genes are used for colonization of the endo niche and the functional properties of the endophytes toward the host. Moreover, better understanding of the relative mechanisms between host plant and endophyte interaction will come to light by the genomic analysis. Genome annotation and comparative genomics allowed the identification of an extended set of genes specific to the plant niche adaptation of the bacterium *Enterobacter* sp. 638, an endophytic plant growth-promoting gammaproteobacterium isolated from the stem of poplar (*Populus trichocarpa6deltoides* cv. H11-11), a potentially important biofuel feedstock plant (Taghavi et al. 2010). The *Enterobacter* sp. 638 genome sequences reveal the presence of a 4,518,712 bp chromosome and a 157,749 bp plasmid (pENT638-1). It further reports genes that code for putative proteins involved for survival in the rhizosphere (to cope with oxidative stress or uptake of nutrients released by plant roots), root adhesion (pili, adhesion, hemagglutinin, cellulose biosynthesis), colonization/establishment inside the plant (chemotaxis, flagella, cellobiose phosphorylase), plant protection against fungal and bacterial infections (siderophore production and synthesis of the antimicrobial compounds 4-hydroxybenzoate and 2-phenylethanol), and improved poplar growth and development through the production of the phytohormones (indole acetic acid, acetoin, and 2,3-butanediol). Interestingly, both the genetic determinants required for sucrose metabolism and the synthesis of acetoin and 2,3-butanediol are clustered on a genomic island. These findings point to a close interaction between *Enterobacter* sp. 638 and its host, where the availability of sucrose, a major plant sugar, affects the synthesis of plant growth-promoting phytohormones by the endophytic bacterium. The availability of the genome sequence, combined with metabolome analysis, will provide a better understanding of the synergistic

interactions between plants and their growth-promoting endophytes (Taghavi et al. 2010). Hence more genome data availability will further update the existing understanding and functioning of plant-microbe interactions and their role in various stress tolerance mechanisms.

Endophyte-Associated Plants: Host to Bioactive Natural Products of Benefits to Host Defense

Natural products are metabolites or by-products from microorganisms, plants, or animals (Baker et al. 2000). Metabolites bearing antibiotic activity can be defined as low-molecular-weight organic natural substances made by microorganisms that are active at low concentrations against other microorganisms (Guo et al. 2008). Bioactive natural compounds produced by endophytes have been a promising potential source of concern (Table 7.1). Development of drug resistance in human pathogenic bacteria, fungal infections, and life-threatening viruses claims a new therapeutic agent for effective treatment of diseases in human, plants, and animals concern that are currently unmet (Zhang et al. 2005). Many endophytes are members of common soil bacterial genera, such as *Pseudomonas*, *Burkholderia*, and *Bacillus* (Lodewyckx et al. 2002). These genera are well known for their diverse range of secondary metabolite products including antibiotics, anticancer compounds, volatile organic compounds, and antifungal, antiviral, antioxidant, insecticidal, and immunosuppressant agents. Additionally, a broad variety of bioactive secondary metabolites with unique structures, including alkaloids, benzopyranones, chinones, flavonoids, phenolic acids, quinones, steroids, terpenoids, tetralones, xanthones, and others (Tan and Zou 2001), have been associated with endophytes. A member of the plant-associated fluorescent pseudomonads, *Pseudomonas viridiflava*, which has been isolated on and within the tissues of many grass species, was found to produce novel antimicrobial compounds called ecomycins (Miller et al. 1998). Volatile compounds obtained from endophytes possess not only sensory properties but other desirable properties such as antimicrobial (vanillin, essential oil constituents), antifungal, antiviral (some alkanolides), antioxidant (eugenol, vanillin), somatic fat reducing (nootkatone), blood pressure regulating (2-[E]-hexenal), anti-inflammatory (1,8-cineole), and others (Firakova et al. 2007).

Cryptosporiopsis quercina is the imperfect stage of *Pezicula cinnamomea*, a fungus commonly associated with hardwood species in Europe. *C. quercina* demonstrated excellent antifungal activity against some important fungal pathogens such as *Candida albicans* and *Trichophyton* spp. (Li et al. 2000). Chaetomugilin A and D with antifungal activities were isolated from an endophytic fungus *Chaetomium globosum* collected from *Ginkgo biloba* (Qin et al. 2009). "Hypericin" (C30H16O8), a naphthodianthrone derivative, and "Emodin" (C15H10O5) have antimicrobial activity against several bacteria and fungi, including *Staphylococcus aureus* sp. *aureus*, *Klebsiella pneumoniae* sp. *ozaenae*, *Pseudomonas aeruginosa*, *Salmonella enterica* sp. *enteric*, and *Escherichia coli*, and fungal organisms *Aspergillus niger* and *C. albicans* (Kusari et al. 2009). *Cladosporium* sp., displaying the most active antifungal activity, was investigated and found to produce a secondary metabolite known as "brefeldin A" (C16H24O4), a lactone with antibiotic activity. "Pumilacidin" is an antifungal compound, by *Bacillus pumilus*. The compounds "2-hexyl-3-methyl-butanodioic acid" and "cytochalasin D" were isolated from the endophytic fungus *Xylaria* sp. from Brazilian Cerrado, with antifungal activities (Cafeu et al. 2005).

Role of Endophytes in Agricultural Applications

Endophytes are increasingly being exploited for their wide application in agriculture increasing the overall food production and reducing the disease incidence by building plant immune system response. The interaction between the host plant and the harmless endophyte on prospecting and protecting the plant from both abiotic and biotic stress factors is studied by many researchers.

Table 7.1 Some natural active products obtained from endophytes

Endophyte	Host	Active metabolites	Target pathogens/ activity	References
Acremonium zeae	Maize	Pyrrocidines A, B	*Aspergillus flavus*, *Fusarium verticillioides*	Wicklow et al. (2005)
Pseudomonas viridiflava	Grass	Ecomycins B and C	*Cryptococcus neoformans*, *C. albicans*	Miller et al. (1998)
Cytonaema sp.	*Quercus* sp. 103	Cytonic acids A and D	*Cytomegalovirus*	Guo et al. (2000)
Muscodor albus	Tropical tree	Tetrahydrofuran, 2-methyl furan, 2-butanone, aciphyllene	*Stachybotrys chartarum*	Atmosukarto et al. (2005)
Streptomyces griseus	*Kandelia candel*	Antimicrobial, p-amino acetophenonic acids	Antimicrobial	Guan et al. (2005)
Streptomyces NRRL 30562	*Kennedia nigriscans*	Munumbicins, munumbicin D	*Bacillus anthracis*, *M. tuberculosis*, *Plasmodium falciparum*	Castillo et al. (2002)
Periconia sp.	*Taxus cuspidata*	Fusicoccane diterpenes	*Bacillus subtilis*, *Staphylococcus aureus*, *Klebsiella pneumoniae*, *Salmonella typhimurium*	Kim et al. (2004)
S. marcescens	*Rhyncholacis penicillata*	Oocydin A	*Pythium ultimum*, *Phytophthora parasitica*, *Phytophthora cinnamomi*, *Phytophthora citrophora*	Strobel et al. (1999), (2004)
Acremonium zeae	Maize	Pyrrocidines A, B	*Aspergillus flavus*, *Fusarium verticillioides*	Wicklow et al. (2005)
Streptomyces NRRL 30566	*Grevillea pteridifolia*	Kakadumycins	*Bacillus anthracis*, *M. tuberculosis*, *Plasmodium falciparum*	Castillo et al. (2003)
Verticillium sp.	*Rehmannia glutinosa*	Massariphenone, ergosterol peroxide	*Pyricularia oryzae* P-2b	You et al. (2009)
Paenibacillus polymyxa	Wheat, lodge pine, green beans, *Arabidopsis thaliana*, canola	Fusaricidin A–D	*Corynebacterium fimi*, *Escherichia coli*, *Leptosphaeria maculans*	Beck et al. (2003), Li et al. (2007), and Beatty and Jensen (2002)
Phomopsis cassiae	*Cassia spectabilis*	Cadinane sesquiterpenes	*Cladosporium sphaerospermum*, *Cladosporium cladosporioides*	Silva et al. (2006)
Ampelomyces sp.	*Urospermum picroides*	3-O-Methylalaternin, altersolanol A	*Staphylococcus aureus*, *S. epidermidis*, *Enterococcus faecalis*	Aly et al. (2008)
Cryptosporiopsis quercina	*Tripterygium wilfordii*	Cryptocin	*Pyricularia oryzae*	Li et al. (2007)
Pestalotiopsis microspora	*Terminalia morobensis*	Pestacin, isopestacin	Antioxidant	Strobel (2002)

The mechanism by which endophytes colonized and localized their host plant and the process through which they confer resistance to attack by broad phytopathogens are a promising area to explore.

As Biocontrol Agents

The reduction in food productivity worldwide is an alarming issue in the present scenario. One of the major causes is diseases of plants that hinder crop production. Therefore, management of diseases by means of harmless bioagents is important. The adaptation of colonizing the internal host tissue has made endophytes more valuable for agriculture, as a tool to improve crop performance and most importantly encourage organic farming, when maximum percent of the agro market is flooded with chemical-based food crops. Many endophytes have mutualistic relationships with their host plant; they obtain nutrients and in turn provide protection to the host from biotic and abiotic stresses. Controlling pests, insects, and phytopathogenic microbes by using anti-ecological chemical compounds like insecticides, fungicides, and herbicides results in the elimination of important species of insects and microbes that control other pests and harmful microbes, performing a crucial role in the environment. Beneficial endophytes are also getting affected, and hence a revolution is essential for preserving such beneficial microbes. The role of endophytes as upcoming biocontrol agents is an area to focus so as to utilize the alternative biosource agents in reducing the use of chemical hazards in agriculture and promoting an ecofriendly substitute.

Against Phytopathogens

Natural products from endophytic microbes have been observed to inhibit or kill a wide variety of harmful disease-causing agents, but not limited to phytopathogens, as well as bacteria, fungi, viruses, and protozoans. Endophytes colonize an ecological niche similar to that of the phytopathogens and hence can be utilized as agents for the control of pathogenic microbes. Endophytic strains of *Microbispora rosea* subsp. *rosea* and *Streptomyces olivochromogenes* were found to be effective in suppressing the club root of Chinese cabbage caused by *Plasmodiophora brassicae* (Lee et al. 2008). Cryptocin, a unique tetramic acid, is also produced by *C. quercina* (Li et al. 2000). This unusual compound possesses potent activity against *Pyricularia oryzae* as well as a number of other plant-pathogenic fungi. An endophytic strain of *Actinoplanes missouriensis* isolated from lupin roots was effective in suppressing the root rot of lupin caused by *Plectosporium tabacinum* (El-Tarabily 2003). Cytosporone B and C were isolated from a mangrove endophytic fungus, *Phomopsis* sp., inhibiting the growth of *C. albicans* and *Fusarium oxysporum* (Huang et al. 2007). Chlorinated metabolites such as (−) mycorrhizin A and (+) cryptosporiopsin isolated from endophytic *Pezicula* strains were reported as strongly fungicidal, herbicidal, and antibacterial agents (Huang et al. 2007). Benzophenone derivatives, "Pestalachlorides A" (C21H21Cl2NO5) and "B" (C20H18Cl2O5), from the plant endophytic fungus *Pestalotiopsis adusta*, display significant antifungal activity against three plant pathogenic fungi, *Fusarium culmorum*, *Gibberella zeae*, and *Verticillium albo-atrum* (Li et al. 2008). Two new bioactive metabolites, "ethyl 2, 4-dihydroxy- 5, 6-dimethylbenzoate" and "phomopsilactone," were isolated from an endophytic fungus *Phomopsis cassiae* from *Cassia spectabilis* and displayed strong antifungal activity against two phytopathogenic fungi, *Cladosporium cladosporioides* and *Cladosporium sphaerospermum* (Silva et al. 2005). There are reported compounds such as amphisin, 2,4-diacetylphloroglucinol (DAPG), hydrogen cyanide, oomycin A, phenazine, pyoluteorin, pyrrolnitrin, tensin, tropolone, and cyclic lipopeptides produced by pseudomonads (Raaijmakers et al. 2002) and oligomycin A, kanosamine, zwittermicin A, and xanthobaccin produced by *Bacillus*, *Streptomyces*, and *Stenotrophomonas* spp., with antimicrobial activities. It is important to note that many strains produce a pallet of secondary antimicrobial metabolites and conditions favoring one compound may not favor another (Duffy and Défago

1999); thus, the varied arsenal of biocontrol strains may enable antagonists to perform their ultimate objective of pathogen suppression under the widest range of environmental conditions. For example, in *Pseudomonas fluorescens* CHA0 biosynthesis of diacetylphloroglucinol (DAPG) is stimulated, and pyoluteorin is repressed in the presence of glucose as a carbon source. As glucose is depleted, however, pyoluteorin becomes the more abundant antimicrobial compound produced (Duffy and Défago 1999).

Against Insect Pests

Recent studies showed that some plant lectins were toxic to sap-sucking insect pests. For example, the lectin from snowdrop (*Galanthus nivalis* agglutinin) is toxic to plant hoppers by binding to gut epithelium and then passing into the hemolymph of a pest (Fitches et al. 2001). The mannose-binding *Pinellia ternata* agglutinin (PTA) gene was also found with significant insecticidal activities against Homopterans (Zhang et al. 2003). Insect bioassays showed that transgenic plant expressing PTA lectin gene significantly inhibited aphid growth and exhibited considerable insecticidal activities against rice brown plant hopper. Population of aphids was smaller in plants infected with recombinant endophyte, *Chaetomium globosum* YY-11 (with antifungal activities) with PTA gene cloned into YY-11 mediated by *Agrobacterium tumefaciens* than in wild-type endophytic fungi-treated plant and controls. It significantly inhibited the growth and reproduction of *Myzus persicae* (green aphid) through endophytes as expressing systems for delivery of insecticidal proteins in plants and enhances resistance of plants to insect pathogens (Qi et al. 2011). Alkaloid produced by endophytes can significantly reduce herbivores (Clay and Schardl 2002). For instance, some endophytes can produce toxic alkaloids such as *Neotyphodium coenophialum*, which exist inside tall fescue *Schedonorus phoenix* (Scop.) *Holub* and greatly limit animal feeding and reproductive performance. In banana, naturally occurring endophytic *F. oxysporum* antagonized nematode *Radopholus similis* in vitro through the production of nematode-antagonistic metabolites (Athman et al. 2006), and inoculation of the endophytes into tissue-cultured plants resulted in improved plant growth and reduced nematode densities.

Against Weeds

Endophytes especially *Neotyphodium* are studied for the ability to protect grasses from other destructive weeds and invading pathogens. Much work has been done on perennial ryegrass and several species of fescue to understand better the mechanism of its defense. Ohio State University demonstrated that fungal endophytes can provide the necessary arsenal for the grass plants to compete more effectively against common weed species. Further exploration on the competitive ability of endophyte-containing grasses can substantially contribute to reduction in weed invasion. Endophyte-containing tall fescue and perennial ryegrass are often better and able to compete against common weed species. Endophyte-containing grasses have greater seed survival, germination, and establishment than cultivars that lack endophytes. The presence of endophyte in perennial ryegrass resulted in a significant decrease in white clover. Endophyte-containing perennial ryegrass maintained greater cover under severe insect stress, whereas those lacking endophytes were more heavily invaded by weeds (Grewal and Richmond 2010).

Role in Detoxification and Degradation of Pathogen Virulence Factors

Another mechanism of biological control is the detoxification of pathogen virulence factors, for example, certain biocontrol agents are able to detoxify albicidin toxin produced by *Xanthomonas albilineans* (Walker et al. 1988; Zhang and Birch 1997). The detoxification mechanisms include production of a protein by an endophyte such as *Klebsiella oxytoca* and *Alcaligenes denitrificans* that reversibly binds with the toxin, (Walker et al. 1988), as well as an irreversible detoxification of albicidin, mediated by an esterase that occurs in *Pantoea dispersa*

(Zhang and Birch 1997). Several microorganisms, including strains of *Burkholderia cepacia* and *Ralstonia solanacearum*, can also hydrolyze fusaric acid, a phytotoxin produced by various *Fusarium* species (Toyoda et al. 1988).

Endophytes and Siderophores

Iron is one among the essential elements required for the sustenance of life. Its availability in the environment is limited; therefore, the scarcity of bioavailable iron in soil habitats and on plant surfaces foments a furious competition (Loper and Henkels 1997). There are certain groups of microbes that help in accumulating iron from the surrounding environment by producing iron-chelating compounds known as siderophores. Various bacterial siderophores differ in their abilities to sequester iron; in general, they deprive pathogenic fungi of this essential element since the fungal siderophores have lower affinity (Loper and Henkels 1999). Therefore, endophytes with high siderophore activity actively colonize the host and protect it from invading pathogens depriving them of the essential micronutrient. Other benefits of using endophytes may include increased nutrient uptake (Malinowski et al. 2000), probably faster than the competing flora so as to overcome and resist the activity of invading pathogens.

Lytic Enzymes and Disease Suppression Mechanisms

The enzymatic activity of enzymes such as chitinase, protease, and glucanase as a defense mechanism is known and studied for applied purposes. Chitinase produced by *Serratia plymuthica* C48 inhibited spore germination and germ tube elongation of *Botrytis cinerea*. The ability to produce extracellular chitinase is considered crucial for *Serratia marcescens* to act as an antagonist against *Sclerotium rolfsii* (Ordentlich et al. 1998). It has also been demonstrated that extracellular chitinase and laminarinase synthesized by *Pseudomonas stutzeri* digest and lyse mycelia of *Fusarium solani* (Lim et al. 1991). The β-1,3-glucanase synthesized by *Paenibacillus* sp. strain 300 and *Streptomyces* sp. strain 385 lyses fungal cell walls of *F. oxysporum* f. sp. *cucumerinum* (Singh et al. 1999). Similar to siderophores and antibiotics, regulation of lytic enzyme production (proteases and chitinases in particular) involves the GacA/GacS (Sacherer et al. 1994) or GrrA/GrrS regulatory systems (Ovadis et al. 2004) and colony phase variation (Lugtenberg et al. 2001).

Host Resistance Induction Mechanism

Certain bacteria trigger a phenomenon known as induced systemic resistance (ISR) phenotypically similar to systemic acquired resistance (SAR). SAR develops when plants successfully activate their defense mechanisms in response to primary infection by a pathogen, notably when the latter induces a hypersensitive reaction through which it becomes limited in a local necrotic lesion of brown desiccated tissue (Van Loon et al. 1998). Several bacterial traits (i.e., flagellation, production of siderophores and lipopolysaccharides) have been proposed to trigger ISR (Van Loon et al. 1998), but there is no compelling evidence for an overall ISR signal produced by bacteria (Haas et al. 2002). ISR is effective against different types of pathogens but differs from SAR in that the inducing bacterium does not cause visible symptoms on the host plant (Van Loon et al. 1998). Bacterial endophytes and their role in ISR have been reviewed by Kloepper and Ryu (2006).

Prospect of Endophyte in Plant Growth Promotion

There are numbers of mechanisms by which bacteria promote plant growth and health. The production of plant growth phytohormones like auxins, cytokinins, gibberellins, and ethylene by an endophyte has been studied. Endophytes are known to produce phytohormones that play an important role in promoting root elongation and

plant growth (Li 2005). Nejad and Johnson (2000) colonized an endophyte into seeds and succeeded to improve seed germination rate and seedling length of rape and tomato, but also significantly reduced virus disease symptoms caused by *Catheter wither*. Endophytic actinomycetes have been reported to produce plant growth-promoting compounds such as IAA and siderophores in vitro (De Oliveira et al. 2010).

Plant Growth Hormones and Ability to Fix Nitrogen

Endophytes can promote plant growth without requiring the use of chemical fertilizers. The mechanism and efficiency of the growth promotion effect depends strongly on the species or endophytic isolate, the host plant, and the prevailing environmental condition from where the host is obtained. Plant-associated microorganisms such as fungi and bacteria evolved biochemical pathways, resulting in the production of plant growth hormones reportedly auxins, absicins, ethylene, gibberellins, and kinetins (Goodman et al. 1986). Volatile substances such as 2,3-butanediol and acetoin produced by bacteria seem to be newly discovered mechanisms responsible for plant growth promotion (Ryu et al. 2003). Plant growth promotion has also been caused by endophytic yeast due to production of auxins, as in the case of *Williopsis saturnus* in maize roots (Nassar et al. 2005). *Piriformospora indica* is another root endophyte that has been shown to promote the growth of both food crops (e.g., maize) and shrubs (*Artemisia annua* L.) with increases up to 50 % of their fresh biomass (Varma et al. 1999). It has also been shown to promote the formation of adventitious roots in cuttings of *Pelargonium* and *Poinsettia* (Drüge et al. 2007). Endophytes produce adenine ribosides that stimulate growth and mitigate browning of pine tissues (Pirttilä et al. 2004). The nitrogen transfer improved host uptake of phosphorus conferred by the endophytic fungus *Cladorrhinum foecundissimum* in cotton roots and increased plant height by 50 % compared with control plants (Gasoni and Stegman de Gurfinkel 1997). Many studies demonstrated that plants infected with endophyte obtain growth promotion (Ait Barka et al. 2002) resistance to drought, stress (Swarthout et al. 2009), and tolerance to unsuitable soil conditions (Malinowski et al. 2004). The enhancement of plant growth may be influenced by compounds like phytohormones produced by fungal endophytes, *Colletotrichum* sp., and endophytic fungus in *A. annua* produces substances like indole acetic acid (IAA) to regulate plant processes (Lu et al. 2000). Rhizobia are very well-known endophytes able to fix nitrogen in the roots of legume plants. The rhizobium-legume system not only provides nitrogen to the legume crop but also enhances the fertility of soil where they are grown. In fact legumes because of their endophytic partner are considered as the most important biological system to enhance the nitrogen content of the soil and hence preferred in crop rotation throughout the globe. Rhizobia are also known as very good phytohormone (especially indole acetic acid) producers which result in root hair proliferation and growth enhancement. Therefore the impact of plant growth is correlated with the production of phytohormones with the supplement of favorable growth conditions.

Stress Tolerance Mechanisms of Endophytes

The fact that plants are continuously exposed to a wide range of environmental stress conditions (comprising both biotic and abiotic factors) is unavoidable, and the need to protect plants from such extreme conditions is vital to preserve the plant life and its yield as it is a serious threat to agriculture. The mutual relation between the host plant and the endophyte significantly improves tolerance to stress factors including drought, high soil salinity, extreme soil pH, insect pests, and nematodes, along with increased persistence and vigor, and in turn the plant provides the symbiont with nutrients, protection, and reliable and efficient dissemination (Schardl et al. 2004). It has become apparent that some plants are unable to tolerate habitat-imposed abiotic and biotic stresses in the absence of fungal endophytes (Redman et al. 2002). Therefore biotechnological

intervention on implementing endophytes for improving plant immunity and health is indeed an important and essential area of study.

Abiotic Stress Tolerance

The study on endophytes for tolerance to extreme temperatures, salinity, pH, flood and drought, and also enhanced immunity of the host plant is now being carried out. The primary cause of crop loss worldwide, reducing average yields for most major crop plants by more than 50 %, is due to abiotic stresses (Bray et al. 2000). Abiotic stress leads to series of morphological, physiological, biochemical, and molecular changes that adversely affect plant growth and productivity (Wang et al. 2001). There seems to be a vital interaction between the endophyte and host, and the resultant chemistry induces plant protection. Secondary compounds produced by endophytic fungi may be responsible for the resistance to biological and abiotic stresses and improvement in the host competitiveness. Secondary metabolites play an important role in resisting drought. Loliterm B, for instance, can alter osmotic potential to reduce the harmful effect of drought (Yuan et al. 2010). Interestingly, Hasegawa et al. (2005) discovered that tissue-cultured seedlings of mountain laurel harboring endophytic *Streptomyces padanus* became highly tolerant to drought. In tolerant seedlings, callose deposition and lignification of cell walls as well as osmotic pressure of cells were accelerated, suggesting that all these events could be closely associated with enhanced drought tolerance. *P. indica* in salt stress conditions increased the tolerance of salt-sensitive barley (*Hordeum vulgare*) cultivar to severe salt stress. *P. indica*-colonized plants contained higher ascorbate concentrations in roots compared with non-colonized plants, while the ratio of ascorbate vs. dehydroascorbate was not significantly altered, but catalase, ascorbate peroxidase, glutathione reductase, dehydroascorbate reductase, and monodehydroascorbate reductase activities were increased (Lamabam et al. 2011). Plant responses to abiotic stresses are complex, involving signal reception and transduction followed by genetic and physiological responses. It is perceived that all plants are capable of perceiving and responding to stress (Bartels and Sunkar 2005). Likewise endophytes with growth potential under stress conditions can be exploited for use in agricultural wastelands for encouraging plantation.

Conclusion

The time is right to explore these nature-friendly microbes and update their various applications. Can endophytes give a point solution to the alarming food crisis too? In terms of agricultural prospect, it's high time to give more emphasis on technology transfer to fields rather than to confine in laboratories or greenhouse study alone. There are interesting twists and challenges in the study of endophytes, the most important being the isolation process, especially the surface sterilization techniques and identification of true absolute endophytes, ignoring the rest of the microbes. In case of thin plant parts, where removal of the outer tissue portion is not possible, chances of non-endophytic microbial growth may be encouraged following the growth of injured cells, after exposure to favorable conditions. Alternatively, some endophyte growth may also be hindered in such cases due to the penetration of chemicals in the tissues. Hence, proper measures and precautions for isolation are important criteria based on plant type and tissues in particular. Schematic representation of endophyte isolation and in vitro evaluation of different biochemical enzyme assays and biocontrol activity against plant pathogens is shown in Fig. 7.1.

One of the frequently sought issues is that will a single potential endophyte actively overcome the existing problems by acting as a biocontrol agent or as a source to novel metabolites or impart protection from various abiotic stresses? Adding to this the potential isolate with positive response when inoculated into its own plant or other plants in field may not yield similar results as were visible in lab conditions. Understanding plant-microbe interactions will surmount the critical process that an endophyte delivers for host benefit. Another view is, though we may get information on the total endophytic microbiome

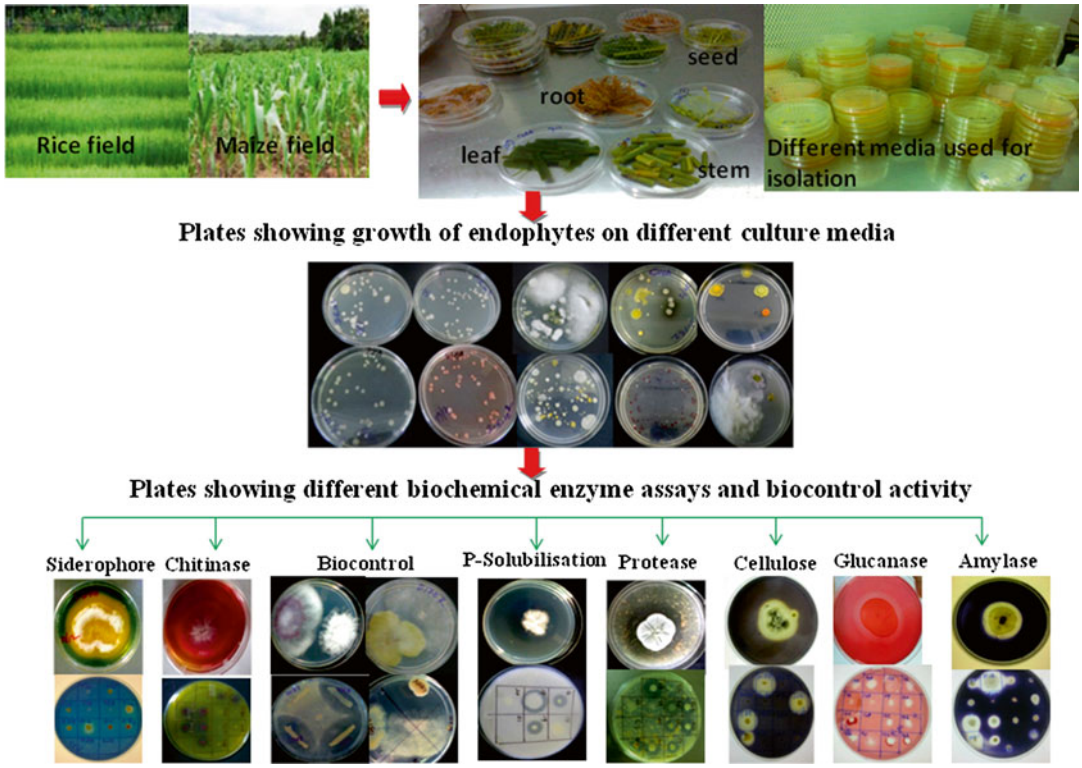

Fig. 7.1 Isolation and in vitro evaluation of different biochemical enzyme assays and biocontrol activity of endophytes

of the host by approaching much advanced high-throughput techniques of culture-independent methods for isolation, how far will we be successful in harboring the most potent microbes, for application is not clear at present. The microorganisms isolated with culture dependence are for sure adaptable to any changes in the environment and hence grow easily without complexity rather than microbes obtained by culture-independent methods. These fragile isolates may find it hard to grow in laboratory and hence difficult to take to the fields as inoculants. Endophytes can readily be termed as "probiotics of plant origin". The survival chain of endophyte involves several animals and human consumers too. Hence, another question arises on endophytes existence in connection to the human microbiome and its overall contribution to human health. Encouring endophyte research and exploitation is essential to answer all the queries. Therefore, discovering the wild and much unexplored territories of multifunctional endophytes and their interaction with the plant host is undoubtedly essential to know more and gain more for a better tomorrow.

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8 Abiotic and Biotic Plant Stress-Tolerant and Beneficial Secondary Metabolites Produced by Endophytic *Bacillus* Species

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Abstract

Knowledge of endophytic bacteria and their potential for protecting crops has targeted the endophytic species of *Bacillus* as a valued microorganism not only for disease protection but also for inducing plant defense mechanisms. *Bacillus* species and their endophytic strains are also used for a wide range of antibiotics that inhibit pathogens directly impacting cellular structures or at the molecular and physiological levels. The endophytic species and strains produce fungal inhibitory compounds that belong to three broad families of lipopeptides, and these include the bacillomycins, fengycins, and surfactins. Bacilli also produce the ribosomally synthesized antimicrobial peptides, bacteriocins, which have been implemented in plant protection schemes to control fungal and bacterial diseases. Others have yet to be identified. These compounds form the basis of intense activity ranging from acute toxicity to serving as signal transduction systems for specific cellular functions, organelle formation, and responses to environmental changes and challenges. This review addresses some evidence of endophytic bacillus impacts on alleviating plant stresses, both abiotic and biotic with suggestions on future studies necessary for specific mechanisms that may assist in increasing their performance as biocontrol agents.

Introduction

The objective of sustainable agriculture is to reduce the industrial applications for food production and replace it with techniques that use fewer chemicals, designed not to harm the environment, ensuring future generations safe and continued use of the agricultural environment. Biological control is often touted as being a system insuring most of the benefits anticipated from sustainable agriculture. There are several

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biocontrol systems being used for specific aspects of agricultural management, and there are many organisms that are being tested along and in combination as biocontrol agents. Leading the list are several genera of bacteria with many life habits. There are species of bacteria that utilize the rhizosphere, the soil, the outside foliage, and the inside, referred to as endophytes. Only a few of these are obligate plant dwellers but nevertheless make useful allies for biocontrol of several plant pests. Others are somewhat obligate and tend to be found on plants naturally. One such group includes the microbial endophytes.

Microbial endophytes include both fungi and bacteria that dwell within plant tissue, and these two groups consist of a large diversity of organisms. This group of microbes was recognized by Galippe (1887) as being distinct from pathogenic species of microbes although he used the term "endophyte" to refer to internal infections of a healthy plant by microorganisms which was used earlier to describe infections with pathogenic species by De Bary (1866) some two plus decades earlier. The term is presently used to refer to microorganisms that dwell within tissues of plants forming usually lifelong associations producing very little harm, and in most instances mutualistic expressions are observed. There are both fungal and bacterial species that form this type of association. Many plants colonized by species in this group are often nonculturable requiring specific molecular techniques for their identification, and even then, identifications often fail. Such obligate strains or species apparently have evolved with the wild ancestors within the plant group. During isolation from related free-living species, they have become distinct and highly dependent on plants for specific growth requirements and vice versa. The native plant hosts show no signs of conflict during the association and often fail to live without the infecting organisms. Scientists are aware of the positive value of endophytic associations, and microbial endophytes are being moved into modern and highly hybridized agricultural cultivars.

Current studies indicate that all plants as their natural communities consist of several endophytic organisms, and as will be discussed later, this might be a rationale for inconsistencies of biocontrol performance when an endophyte is managed in crop plants under controlled agricultural habitats. Microbial endophytes offer several advantages over non-endophytic biocontrol species. One, the major advantage that this group offers is an easy procedure for infections into target plants since studies show that they either self-infect through broken roots or infect during seed germination hence not requiring several field applications during crop development. Another advantage is internal growth in the host, colonizing the tissue as the plant grows. We include in the desirable list of bacterial endophytes those that do not invade the plant cells, especially the xylem, but remain intercellular but whose habitat might be localized in the roots and/or the above ground foliage of the plant.

Due to the endophytic habit and genetic diversity, bacterial endophytes have the potential for a variety of uses such as phytoremediation, biological control of pests, and agronomic enhancements of crop plants' growth and maturity. A recent thrust area is the use of bacterial endophytes to reduce biotic and abiotic stresses. Bacterial endophytes are largely being exploited for disease and pest controls primarily because of their ability to produce a variety of secondary metabolites many of which are antibiotics. However, the knowledge of the multitude of compounds and their specific attributes is only suggestive since very few of these compounds have been demonstrated in situ. Decades of studies designed to describe the efficient use of a biocontrol organism have not developed into a successful strategy. We have not defined all the necessary requirements and interactions that are reflective of many unknown mechanisms that may be operative.

Of the many species of bacteria that have been isolated from plants as endophytes, some species offer more promise due to their unique characteristic. The genus *Bacillus* consists of several spore-forming species with additional features of being natural soil dwellers with the genetic potential of undergoing cell differentiation,

forming spores under stress, but returns to the vegetative state when conditions are favorable. Signal transduction is considered the basic mechanism through which the vegetative and spore states are initiated (Fuqua and Greenberg 2002). *Bacillus* species are often isolated as endophytes, and since they produce very resistant dormant endospores, storage and keeping qualities are not an issue; hence there are numerous convenient commercial procedures for preserving and extending their shelf life. Indeed, viable species of *Bacillus* have been recovered from viable spores after years of storages from most environments (Kennedy et al. 1994; Potts 1994), and there is one report of the recovery of a viable *Bacillus* relatively isolated from a 25-million-year-old amber sample (Cano and Borucki 1995).

In general, *Bacillus* species are generally recognized as safe and the organisms and their products are allowed in human food industry, used as additives in livestock and poultry feed. There are many species of *Bacillus*, both patented and nonpatented, for use as biocontrol agents (Ongena et al. 2007; Ongena and Jacques 2007; Zhou et al. 2008; Raaijmaker et al. 2010; Li et al. 2012; Bacon and Hinton 2014). Considerable discussions will be presented on *Bacillus mojavensis*, a plant-friendly endophytic species that offers several advantages for studies with a strong affinity for a variety of hosts. Strains of this species are desert isolates and exhibit considerable heterogeneity (Olubajo and Bacon 2008). This should translate into a wide range of uses in crop protection such as reduced maize and wheat infections from *Fusarium* species, with the added bonus of mycotoxin reduction produced from fungi of the *Fusarium* species, e.g., deoxynivalenol and the fumonisins.

Due to the large diversity of organisms, and the proposed mechanisms of action that are either contradictory or not observational, we review in this chapter species of the genus *Bacillus* that are considered as endophytic. In some instances and for comparisons, we will detail research from non-endophytic species although endophytic strains of that species have been reported.

Bioactive Substances and Effects on Plants

The foundation for the successful use of bacterial endophytes depends on the antimicrobial and growth-enhancing compounds usually first demonstrated in laboratory. However, demonstration that a *Bacillus* species produce one or several inhibitory substances isolated from culture does not always follow the in planta production. Table 8.1 depicts a list of biologically active compounds that have been isolated from bacilli. The biologically active compounds may be divided into those produced by non-ribosomal synthesis such as the cyclic lipopeptides and to compounds such as the polyketides that are produced by genes or in gene clusters. However, we should indicate first the species of bacteria that have proven useful as biocontrol agents. These species are not necessarily entirely endophytic, although most have strains that have been demonstrated as being endophytic. These species include *B. subtilis*, *B. amyloliquefaciens*, *B. licheniformis*, *B. pasteurii*, *B. cereus*, *B. pumilus*, *B. mojavensis*, *B. mycoides*, and *B. sphaericus*.

Fungicidal and bactericidal compounds have been isolated from most endophytic species, and there are almost similar compounds found throughout the genus, regardless of the species, suggesting an evolutionary strategy based on the importance of the compounds for ecological success within niche diversities. The surfactins apparently occur genus wide, which suggests an important role in the competitive abilities of the species. Others are variations of the surfactin group, although within a species there apparently are strain differences.

Such structural diversity offers more promise for strains within the genus as these variants might prove valuable in selecting strains for specific environments or crop plants with specific problems. Several of the species identified above have been subsequently transferred to a newly erected or related genus, such as *Paenibacillus*, although in this particular genus similar compounds are produced indicating again the importance of these compounds. Demonstration that the *Bacillus*

Table 8.1 Bioactive compounds by *Bacillus* species

Metabolite	Source	Biological activity	References
Bacteriocins	*B. subtilis*	Antagonism against the phytopathogenic fungi, e.g., *Agrobacterium tumefaciens* and *Alternaria solani*	Hammami et al. (2009, 2012), Araújo et al. (2005), and Ashwini and Srividya (2013)
	B. licheniformis	Growth inhibition of fungal pathogens	He et al. (2006)
	B. amyloliquefaciens	Bacterial antagonism to *Ralstonia solanacearum*	Hu et al. (2010)
	B. thuringiensis	Antibacterial and potential plant growth promoter	Lee et al. (2009) and Gray et al. (2006)
	B. clausii	Bactericidal activity against *Agrobacterium tumefaciens*	Mouloud et al. (2013)
Chitinases	*B. amyloliquefaciens*	Fungal growth inhibition	Wang et al. (2002)
	B. licheniformis	Antifungal activity	Gomaa (2012)
	B. thuringiensis	Biocontrol of phytopathogenic fungi	Reyes-Ramirez et al. (2004)
	B. pumilus	Fungal growth inhibition	Shali et al. (2010)
	B. subtilis	Fungal growth suppressor	Ashwini and Srividya (2013)
	B. cereus	Conidial germination inhibitor of *Botrytis elliptica*	Huang et al. (2005)
Plant growth promoters	*B. pumilus*	Indoleacetic acid synthesis for plant growth promotion	Murugappan et al. (2013)
	B. subtilis	Indoleacetic acid and abscisic acid	Araújo et al. (2005)
	B. amyloliquefaciens	Auxin biosynthesis to promote plant growth	Talboys et al. (2014)
Siderophores	*B. pumilus*	Iron sequestration and plant pathogen inhibition	Murugappan et al. (2013)
	B. subtilis	Iron sequestration and plant growth promoter	Yu et al. (2011)
	B. mojavensis	Iron sequestration	Vendan et al. (2010) and Bacon and Hinton (2011a)
Stilbene	*Bacillus* sp.	Antimicrobial activity to phytopathogenic bacteria and fungi	Kumar et al. (2012b)
Phytase	*B. amyloliquefaciens*	Plant growth stimulator under phosphate limitation	Idriss et al. (2002)
Zwittermicin A	*B. cereus*	High antimicrobial activity against oomycetes	Silo-Suh et al. (1998)

species produce one or several inhibitory substances isolated from culture has not been demonstrated *in planta*. Indeed, very little work exists to indicate that such biologically active compounds are produced *in planta*. In addition, the species discussed in this review are not necessarily reported as being endophytic since the techniques of their isolation would not allow for such a distinction.

Lipopeptides

Lipopeptides make up a group of biosurfactants that are closely related metabolites, which include the surfactins, iturins, and the fengycins (plipastatin). This class of metabolites is not produced by ribosomes, rather by a group of peptide synthetases. The lipopeptides, in addition to

producing antimicrobial activity, also act as immune stimulators of the host plant. The antibiotic activity is accomplished by destroying or interfering with cell membranes' properties affecting the pathogen's membrane. However, the surfactins and other lipopeptides are ineffective in penetrating phytosterols, and plant cell membranes consist of phytosterols (Ongena and Jacques 2007). The phytosteroidal composition of host plant cells is chemically sufficient to provide protection from these substances indicating some level of selectivity over pathogens. Thus, both sensitivity and specificity reside in the nature of both the pathogen's cell wall and the host cell wall, and perhaps some pathogens might have cell walls that are resistant to these biosurfactants as well.

Of the many compounds that are indicated above, the cyclic lipopeptides are perhaps the most suitable for in planta crop protection (Ongena and Jacques 2007; Romero et al. 2007; Raaijmakers et al. 2010). For example, the physical characteristics of surfactins increase their benefits as biocontrol agents. This group of lipopeptides is stable over a wide range of conditions. They are active from 15 °C up to 100 °C and are structurally sound over a range of pH values from 3 to 11 (Cameotra and Makkar 1998; Makkar and Cameotra 1998; Joshi et al. 2008). These compounds are tolerant to most plant and microbial enzymes such as the pronases and proteinases and somewhat stable in organic solvents. Due to these properties, the lipopeptides have a wide tolerance of environmental conditions. *Bacillus* species and strains producing a combination of these compounds offer more promise as biocontrol agents for crops as compared to others as lipopeptides are associated with a variety of positive biocontrol activities including biofilm formation, signaling, and fungal antagonisms (Bais et al. 2004; Kearns et al. 2005; Ongena and Jacques 2007).

The wide spectrum of activity is based on the uniqueness of the chemical structure of the lipopeptides. The endophytic habit should allow the complete expression of each chemical group produced under endophytic conditions. However, such control expressions potentially can be negated by *Bacillus* species that are soilborne or are non-endophytes. That is, the soil environment can impose constraints on the production and functional activity of such antibiotics as soil is subjected to variations, some of which exceed the limits indicated above, particularly the stability. However, in the in planta situation, the environment is consistent, and generally conditions are amendable to maximum production of single or multiple lipopeptides some of which have not been identified (Snook et al. 2009).

In addition to the variety of surfactins produced by *B. mojavensis*, the organism shares all the benefits afforded by most *Bacillus* species as discussed. Further, the desert strains are all plant friendly, producing symptomless infection in all tissues except the xylem, and the infection is maintained during the season (Bacon and Hinton 2002, 2011b). This species is an osmophile tolerating seawater and capable of anaerobic growth (Folmsbee et al. 2004).

Most of the strains of this species originated from the deserts and have been studied relative to their protection of maize from species of *Fusarium* (Bacon and Hinton 2002, 2011a) and for their ability to reduce the accumulation of the fumonisin mycotoxins (Bacon et al. 2001). It was determined that most strains of the desert group produced the surfactins (Snook et al. 2009). The variation of the acyl chain length of each isoform varies. Thirty-three strains isolated from the deserts around the globe produced surfactins with carbon length ranging from 11 to 17, with the majority producing a carbon length of 15, which is the most active form of the surfactins (Snook et al. 2009; Bacon et al. 2013). The surfactins produced by each strain vary resulting in the accumulation of several isoforms by each strain, and the combination of several isoforms may be synergistic, extending the overall spectrum of activity. The surfactins are inhibitory to virus, bacteria, fungi, and insects. However, in vitro fermentation capacity of biosurfactants by strains of *B. mojavensis* is rather poor, which limits its use for technological exploitations for surfactin production.

Bacteriocins

Bacteriocins are a heterologous group of small bacterial peptides produced by ribosomal synthesis with antimicrobial activities against Gram-positive and Gram-negative bacteria and fungal pathogens (Jack et al. 1995; Benitez et al. 2010). Many of the bacteriocins of the genus *Bacillus* display high specificity of antimicrobial activities against related species; however, recent evidence suggests that some of these peptides target a wide range of unrelated microorganisms (Abriouel et al. 2011). Compared to bacteriocins produced by lactic acid bacteria, another Gram-positive group, those produced by the bacilli, has not attracted the curiosity of plant scientists. The initial in vitro screenings indicated that *Bacillus* antimicrobial peptides exhibited limited antimicrobial activities and were usually restricted to members of the same species. In the last decade, new evidences have revealed a wide array of proteinaceous bactericides by the genus *Bacillus* with remarkable efficiency and broad antimicrobial spectrum against important plant pathogens (Emmert and Handelsman 1999; Kim and Chung 2004; Balhara et al. 2011).

The characterization of *Bacillus* bacteriocins revealed several traits distinctive of these peptide antibiotics. A number of bacteriocins belong to the group of lantibiotics, a group of heat-stable precursor peptides with characteristic polycyclic thioether amino acids lanthionine and methyllantionine that undergo posttranscriptional modifications (Jack et al. 1995; Willey and van der Donk 2007).

Several bacteriocins from plant-associated bacteria of the genus *Bacillus* have been identified as potential candidates to control important plant pathogenic microorganisms. The strain *B. subtilis* 14B, isolated from the rhizosphere of healthy almond plants from Turkey, is capable of producing a monomeric protein (termed Bac 14B) with a molecular weight of 21 kDa. Bac 14B is a bacteriocin with high antimicrobial activity against *Agrobacterium tumefaciens*, the causal agent of crown gall disease (Hammami et al. 2009). Interestingly, Bac 14B displayed a broader antibacterial spectrum against *Agrobacterium* spp. in comparison to two commercially available biocontrol products for this disease: *Agrobacterium rhizogenes* K84 and *Agrobacterium radiobacter* strain K1026. An endophytic *B. amyloliquefaciens* isolated from mangrove (*Rhizophora mangle*) leaves synthesized a class II bacteriocin with a molecular weight of approximately 10 kDa. This newly characterized bacteriocin displayed inhibition against *Ralstonia solanacearum*, the causal agent of *Capsicum* bacterial wilt (Hu et al. 2010). Although other *B. amyloliquefaciens* bacteriocins are known as fungal inhibitors (Yoshida et al. 2001; Yu et al. 2002), this bacteriocin remarkably displayed antibacterial activity against 2 g of negative plant pathogens *Xanthomonas campestris* and *R. solanacearum*.

Bacteriocins are known for their antimicrobial activity against a wide array of microorganisms. However, it has been suggested that a bacteriocin, thuricin 17, obtained from the plant growth-promoting rhizobacterium *Bacillus thuringiensis* might be involved in the enhanced plant growth in soybean (Gray et al. 2006). Current evidence has also shown that thuricin 17, when applied to either leaves or roots of soybean plants, directly increased plant growth (Lee et al. 2009). While more evidence is needed on the role of thuricin 17 in planta growth promotion, these findings opened a new line of investigation to understand the plant-bacterium associations leading to plant growth promotion by the rhizobacterium, *B. thuringiensis*. Finally, a role for this group of compounds has been suggested in the autoinduction and activation of gene clusters as well as involvement in quorum sensing (Kleerebezem et al. 1997).

Polyketides and Other Defensive Metabolites

Other non-ribosomal antimicrobial secondary metabolites produced by the bacilli include the polyketides. Examples of such metabolites are bacillomycin D, bacillaene, and anticapsin. Most of these are antifungal, antiviral, and anti-mycoplasmal and a few are broad-spectrum antibactericidal. Other secondary metabolites that

exhibit remarkably strong antagonisms to fungi include difficidin, mersacidin, subtilin, subtilosin A, sublancin, and TasA. These compounds are also not produced by ribosome, but are produced by polyketide synthases. Their range of activity also includes toxicity to Gram-negative bacteria, viruses, filamentous fungi, and as presented below bacillibactins that function as siderophores. The major uses of the polyketides are associated with crop protection since their spectrum of activity is restricted to microbial antagonisms.

Bacillus species are a potent source of bioactive secondary metabolites (see Table 8.1) with a variety of functions, including antibiotic activity, enzyme inhibitors, mineral scavengers, and surfactants (Vining 1990; Sansinenea and Ortiz 2011; Makkar and Cameotra 2002). While a great percentage of research has been dedicated to study antimicrobial peptides, current studies suggest that endophytic bacilli and their metabolites might be able to modulate a wide array of plant responses to biotic and abiotic stresses that enable the host to survive under a variety of harsh natural conditions. Ecologically, these observations have been demonstrated on a variety of wild populations with the assumption that they are also operative in many crop plants. This assumption removes the involvement of the host in endophytic relationships, which, however, is probably not the case always.

The endophytes of *B. mojavensis* secrete siderophores as well as do other *Bacillus* species (de Silva et al. 1996; Vendan et al. 2010; Bacon and Hinton 2011a). Siderophores are low molecular weight iron chelators that increase abiotic plant resistance to deficiency of iron, zinc, copper, lead, cadmium, chromium, mercury, and nickel. Included in this group are pyoverdine, pyochelin, and several organic acids. Siderophores enhance either the solubility or uptake of these metals, some of which are important micronutrients for plants and microorganisms. While these substances are present in the soils, they are usually combined with other compounds and not in soluble forms. For example, although iron is among the most abundant micronutrients in agricultural soils, the low solubility of the ferric ion in soil and marine sediments limits iron acquisition by plants. Current data show that siderophore-mediated iron sequestration by endophytic *Bacillus* may confer a competitive advantage to the plant host with respect to pathogen inhibition (Murugappan et al. 2013). In addition to mobilizing forms of micronutrients, microbial siderophores also immobilize some metals preventing them from accumulating to toxic amounts in host plants. Immobilization may be accomplished either through a chemical reduction from one form to a less soluble or mobile form.

It is not certain but future research will determine to what extent siderophore production promotes plant growth, but presently the effects on reducing plant disease should be interpreted as indirect. In greenhouse experiments, *B. subtilis* CAS15 strain isolated from the rhizosphere of pepper fields secreted the catecholic siderophore bacillibactin (2,3-dihydroxybenzoate-glycine-threonine trimeric ester) (Yu et al. 2011). In these studies, the authors demonstrated that increasing the iron concentration resulted in significant decrease in bacillibactin by *B. subtilis*. The plant growth-promoting bacterium, *B. cereus*, isolated as an endophyte from *Sophora alopecuroides* in China, in addition to other compounds, secretes siderophores, suggesting that these molecules might be involved in plant growth promotion (Zhao et al. 2011).

Stilbene phytoalexins are also important bioactive compounds secreted by *Bacillus* sp. that have been associated with increased plant disease resistance (Dercks and Creasy 1989; Paul et al. 1998). Phytoalexins are low molecular weight antibiotics synthesized by plants and bacteria that promptly accumulate in response to biotic or abiotic stress at the infection sites (van Etten et al. 1994; Hammerschmidt and Dann 1999). The stilbene 3,4,5-trihydroxystilbene, known as resveratrol, and 3,5-dihydroxy-4-isopropylstilbene were isolated from ethyl acetate extracts from *Bacillus* strains that showed high antifungal activity against several medical and agriculturally important crop pathogens such as *Ralstonia solani* (Kumar et al. 2012b). Current data show that stilbenes produced by *Bacillus* sp. act synergistically with the antibiotic fluoroquinolone, an important cephalosporin used to control Gram-negative and

anaerobic bacteria (Kumar et al. 2012a). In addition, stilbenes are potentially useful for controlling important plant pathogens such as oomycetes (Kumar and Nambisan 2014), and resveratrol, a stilbene-type phenol, is an important antioxidant used in human medicine for cancer prevention as well as a powerful antioxidant that protects plants from environmental stresses.

Many endophytic bacilli produce plant hormones such as gibberellins, auxin, and cytokinins (Ortiz-Castro et al. 2008; Malfanova et al. 2011; Murugappan et al. 2013). To evaluate the role of cytokinins by the plant-associated bacterium *Bacillus megaterium*, in vitro experiments were performed using *Arabidopsis thaliana* (Ortiz-Castro et al. 2008). Compared to the *A. thaliana* ecotype, and in the presence of the cytokinin-producing *B. megaterium*, *A. thaliana* mutants lacking cytokinin receptors showed reduced plant development. These studies showed that *B. megaterium* cytokinins are an important factor in *A. thaliana* growth. A plant-associated bacterium, *B. pumilus*, synthesizes indoleacetic acid (IAA), the most naturally occurring phytohormone in the auxin class (Murugappan et al. 2013). Similar to cytokinins, IAA produced by plant-associated *Bacillus* was associated with promotion of root growth and increased nodulation in *Phaseolus vulgaris* by *Rhizobium etli* (Srinivasan et al. 1996).

B. cereus and *B. thuringiensis* produce zwittermicin A, a linear aminopolyol antibiotic with broad-spectrum antimicrobial activity against oomycetes and Gram-negative and Gram-positive bacteria (Silo-Suh et al. 1998; Zhou et al. 2008). Structurally, zwittermicin A is unique as it is a combination of a polyketide, a peptide that includes moieties consisting of a D-amino acid, an ethanolamine group, and a glycolyl group. Further, this antibiotic is produced by both polyketide and non-ribosomal biosynthetic pathways (Milner et al. 1996). Studies on the production of zwittermicin A by *B. cereus* revealed that the acetyltransferase gene to catalyze the acetylation of zwittermicin A belongs to a new class of acetyltransferase with self-resistance properties for *B. cereus* providing a novel method of disease protection (Zhou et al. 2008).

In Planta Interactions and Functions of Bioactive Compounds

Knowledge of the actions of one or more mechanisms is required to predict control of target species. This will lead to the dependable and efficient use of endophytic bacteria as biocontrol agents. Very little is known about the specific mechanisms involved, including in planta production, accumulation, and regulation of responsible metabolites. Questions arise, such as can these be regulated under current crop production practices and if not how to modify production practices to achieve the desired result? Very few studies are designed to test if in fact metabolites isolated in culture are produced in planta.

There are several proposed mechanisms for the action of biocontrol bacteria, and these include antimicrobial activity mediated by one or more secondary metabolites such as the production of antibiotics, allelochemicals, nutrient stimulators, niche exclusion, and systemic acquired resistance and the production of volatile secondary metabolites that can be interactive in soils (Ryu et al. 2004). Many have been implicated in the elaboration of specific morphological structuring compounds such as colony surface attachments allowing for multicellular aggregation and attachment to cell surfaces, particularly biofilms. Of importance is their role in mediating cell signaling and quorum sensing. Included in these mechanisms are also those defensive hydrolytic proteins such as hydrolases, chitinases, and lipases (Idriss et al. 2002; Huang et al. 2005; Gomaa 2012). Others involve the detoxification and degradation of various pathogen virulence factors. Certain secondary metabolites produced by bacterial endophytes can also induce systemic acquired resistance.

Prolonged planting of a crop over time or even rotational plantings will result in a group of resistant organisms that dominate within these locations. The result is a group of resistant pathogens to both host natural defenses and possibly to any biocontrol agent. However, there is emerging evidence that some bacteria also are effective modifiers of their host natural defensive compounds such that the host metabolites, often not effective

to resistant pathogens, are modified by their endophytic partner into compounds that are highly effective to control pathogens as well as other competing endophytes within specific crops. Such chemical modifications can be viewed as a control mechanism at the population level since they are competing with one or more pathogenic species naturally associated with crop plantings. For example, it has been observed that *B. mojavensis* chemically modifies a group of maize phytoanticipins, the hydroxamic acids (benzoxazolinones), into the toxic compound 2-amino-3*H*-phenoxazin-3-one (APO) (Bacon et al. 2007), that are toxic to *Fusarium verticillioides* and other hydroxamic acid-resistant pathogens (Fig. 8.1). The hydroxamic acid in spite of being short lived is not an effective inhibitor to the usual maize endophytic pathogenic fungi, the *Fusarium* species and *Diplodia*, which over time can and do dominate fields under constant cultivation of maize. However, these maize pathogens are very susceptible to the bacterial modified compounds, APO, which have the potential for controlling populations of pathogens (Glenn et al. 2001, 2002; Bacon et al. 2007). In a more detailed study, Glenn et al. (2003) have used this as an explanation for the dominance of *F. verticillioides* and other maize pathogens that dominate field under constant maize cultivation. Further, there is some evidence for host genetic modifications indicating a two-point control system (Glenn et al. 2003). Of course, there are converse examples as well. A target pathogen can and does produce a variety of substances and mechanisms that are capable of controlling or nullifying any activity of a biocontrol agent. The wide-scale production of fusaric acid, an antibiotic substance, by *Fusarium* species is one example (Bacon et al. 1996, 2004). Fusaric acid is highly toxic to most bacteria providing some measure of control over a biocontrol bacterium. Thus, there are mutual inhibiting properties that have to be considered, resulting in the degree of success in the field that is only partial from a few biocontrol bacterial endophytes.

Since most of the researches dealing with the production of biologically active compounds by bacterial endophytes, such as antibiotics, have been demonstrated under careful laboratory conditions, very little have been established for in planta production. Experiments designed to test their regulations with or without interaction from the plant physiological processes have not been determined. There is, however, considerable information for increased plant performance by bacterial endophytes when cultured under conditions using a single endophytic microbe co-inoculated with a single pathogen. However, the bacterial endophytes are in competition with all soil and foliage microbes as well as the target pathogen or pest. Clearly, many enhanced effects observed by endophytes and usually reported are not produced along with multiple competing organisms. The point here is if bacterial endophytes are to be tested under laboratory conditions, such multiple infections by naturally occurring organisms should be used in standard procedures which is not the case at present. There are, however, inocula consisting of multiple species or strains used as biocontrol agents.

Abiotic and Biotic Stress-Tolerant Applications

Plants are subjected to a variety of environmental stresses such as soil pH, drought, hot and cold temperatures, air pollution, soil salinity, heavy metal contamination, and soil and foliage parasitism. In an agricultural environment, all these factors affect plant functions, which corresponds to yield reduction to less than optimal. And if excessive, death of the crop is assured. Conceivably each of these abiotic and biotic stresses can be controlled by separate mechanisms based on adaptations of each crop species, its genetic potential to withstand stresses, and the importance of each stress on the physiology of the plant, that is, whether the mechanism affected is a primary process such as photosynthesis or a minor process as production of secondary root development. Distinct from the basic physiology of the plant, there are microorganisms that allow tolerance or increase the tolerance against stresses in the hosts (Warren 1998; Mayak et al. 2004; Vinocur and Altman 2005; Zaidi et al. 2006; Hardoim et al. 2008;

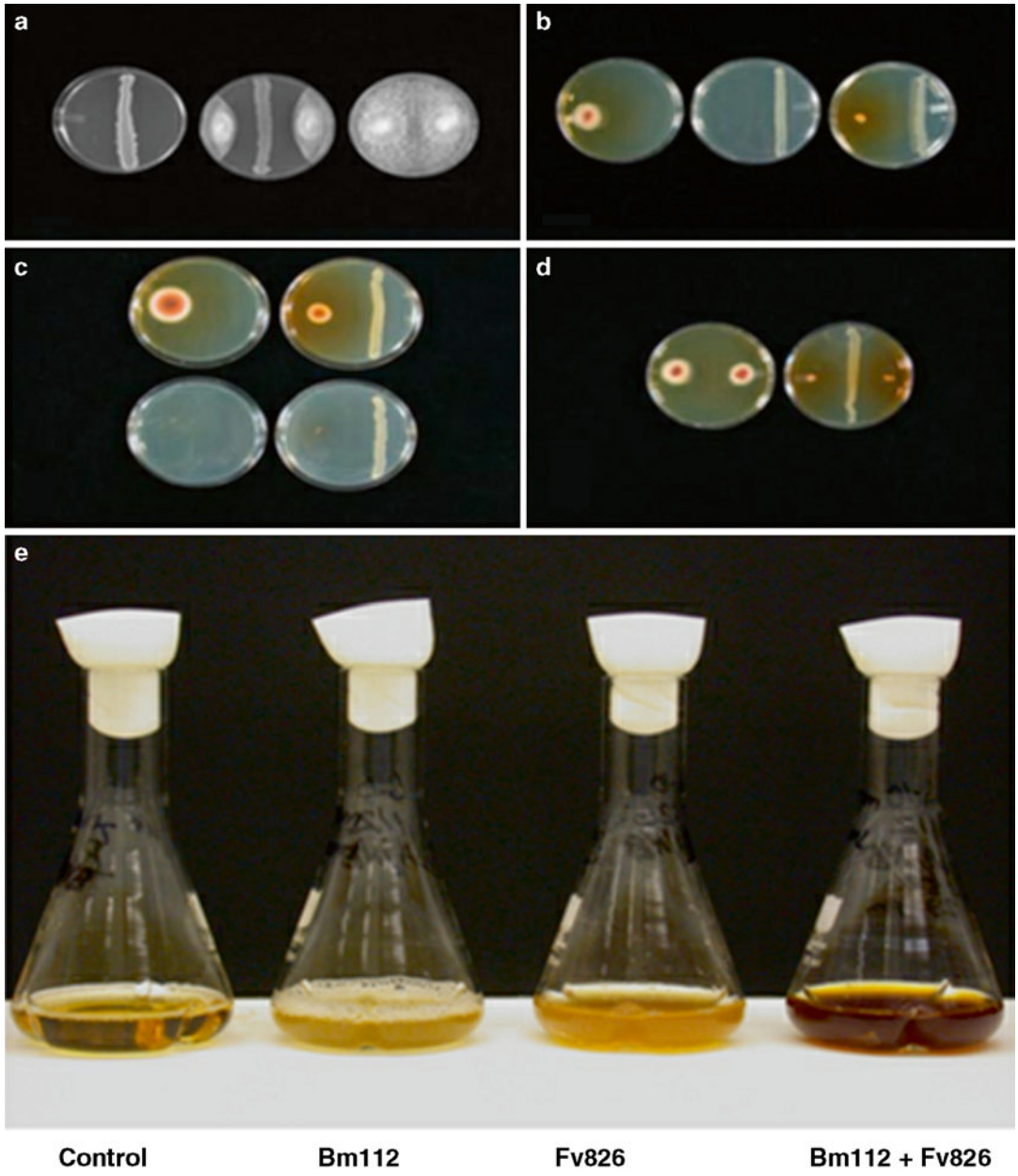

Fig. 8.1 Culture of *Bacillus mojavensis* RRC102 and *Fusarium verticillioides* MRC826 on nutrient agar amended with benzoxazolinones, specifically benzoxazolin-2(3*H*)-one (BOA). (**a**) The zone of inhibition produced by the toxic metabolite transformed from BOA by the bacterium, center plate. (**b**) Culture of *Fusarium verticillioides* cultured with and without *B. mojavensis* on nutrient agar and on BOA-amended nutrient agar showing expression of red pigment, identified as 2-amino-3*H*-phenoxazin-3-one (APO), and severely inhibited on plate far right. (**c**) *Top row*, production of APO, the red pigment, by *F. verticillioides* (RRC 826) with and without the bacterium *B. mojavensis* (RRC 112); bottom row, agar plates showing no growth with and without one of the benzoxazolinone-sensitive genetic strains of *F. verticillioides* with and without the bacterium. (**d**) Production of the red pigment, APO, achieved in the culture in the presence of *F. verticillioides* RRC826 along (*left*) and with the bacterium (*right*). (**e**) Liquid culture of *B. mojavensis* (Bm112) and *F. verticillioides* (Fv826) on nutrient broth amended with BOA resulting in the production of APO during the culture of both organisms (Bm112 and Fv826) (Bacon et al. 2007)

Ryan et al. 2008; Vardharajula et al. 2011; Luo et al. 2012; Yandigeri et al. 2012). A major objective is to increase agricultural production in the land located in unfavorable environments. Traditionally this research is being done by classical genetic and crop breeding techniques. *Bacillus* species have been shown to promote growth in the presence of abiotic stress such as drought. Bacterial endophytes have the potential to induce stress resistance in crops grown under adverse conditions. Endophytes can be used as phytoremediators of soil contaminated with several heavy metals. This approach is novel and recent. The mechanism of action is complex involving several metabolites such as organic acids, along with siderophores resulting in metal detoxification, nutrient acquisition, and often for relief from biotic and abiotic stresses. The process also results in decontamination of heavy metals from plant tissues (Wani et al. 2007; Luo et al. 2012; Rajkumar et al. 2012). While highly desirable, most of this work has not been attempted and is highly theoretical. There is an expected host interaction along with several of its endophytes that will allow the host to grow under environmental extremes (Sziderics et al. 2007; Schalk et al. 2008; Sgroy et al. 2009).

Mixed bacterial populations are also used to provide an array of enhanced effects in a complex environment. Thus, plant hormone-producing bacteria are used along with antibiotic or antagonistic bacteria. Other combinations involve nitrogen-fixing bacteria as well. Apparently, it is also important to determine whether the addition of a single or mixed bacterial population will be beneficial or synergistic for the host. Sturz and Christie (1995) indicated that inhibitory bacteria when added as mixed cultures actually stimulated plant growth. Detailed discussions on the problems and benefits on the uses of mixed cultures of biocontrol bacteria in crop protection are discussed by Stuz et al. (2000).

Conclusion

Bacterial endophytes offer a highly desirable approach for control of plant pathogens. Most of these are self-infecting, co-habitating with the host, and invading the host tissue as the host grows. In spite of the accumulation of antibiotic substances by biocontrol bacterial endophytes, demonstration of their effects under field conditions is inconsistent or lacking. This may be due to unfavorable environmental conditions for the combination being tested; host genetic effect might also compound the results, or competing microbes other than the target microbe might be interfering with colonization or decomposing the inhibitory metabolites. Suitable ecological niches are expected to play a role in biocontrol parameters, and this should occur without doing harm to the host plant. As there are several endophytic species isolated from a host, there is the concept of microniche that should be examined for a functional role in crop production. Acceptance by the host plant could also influence the results, so it is important that only plant-friendly strains be used.

Environmental conditions, especially in the soil, may impose additional stresses on the host, reducing its tolerance to endophytic colonization. Disease recognition and plant induction mechanisms may be required for success with very little to do with specific inhibitory compounds. The mode of action might be based on an induced systemic resistance response. Signaling pathways are expected to be interactive in this regard, resulting in either a positive or no response from the plant host to a disease. The knowledge on basic contribution of a host is lacking although there is an interaction (Smith and Goodman 1999; Smith et al. 1999). The utilization of specific nutrients by the plants or incorporating these in the soil might stimulate the association to produce more of the bioactive compounds. However, to date there are no indications that soil additions will increase positive responses from an endophyte-infected host.

Consistent and effective biocontrol of pathogens will depend on the complex nature of events resulting from the compatible response resulting from plant infection and colonization of tissues by the endophytes. This is followed by the initiation of a cascade of several mechanisms, any one of which if blocked might prevent one or all desired mechanisms necessary for biocontrol.

Overcoming the challenges induced by invading pathogens is monumental; however, successful uses of biocontrol bacterial endophytes are dependent on continued fundamental studies of host-bacterial-pests interactions.

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Antifungal Compounds from Pseudomonads and the Study of Their Molecular Features for Disease Suppression Against Soil Borne Pathogens

9

Urja Pandya and Meenu Saraf

Abstract

The identification and biological and molecular characterization of *Pseudomonas*, a very versatile microbe with biocontrol potential, is of great interest for the modern and eco-compatible agriculture. The mechanisms of biocontrol by *Pseudomonas* include antibiotic production, siderophore production, and production of fungal cell wall-lysing enzymes and induced systemic resistance. Current genome analyses of biocontrol traits will likely lead to the development of novel tools for effective management of indigenous and inoculated pseudomonads as biocontrol agents and a better exploitation of their properties for sustainable agriculture. The chapter summarizes and discusses various studies of pseudomonads from the plant rhizosphere and their use for exploring disease management in integrated disease management (IDM) and the study of genome sequences of *Pseudomonas* spp. for sustainable application.

Introduction

Food security is the one of the most pressing challenges faced currently by humanity. The world population is predicted to reach nine billion by 2050, requiring food production to increase by 50 % against a backdrop of climate change and the loss of arable land. Despite agricultural advances plant pathogens still account for losses of 10–16 % in the global harvest. The need for new control strategies for this and other plant diseases is therefore urgent. At present, the major strategies against damages caused by plant pathogens are chemical pesticides or resistant plant cultivars. However, there are major limitations in using both strategies (Borriss 2011): (1) agrochemicals do not prevent all kinds of diseases, and toxic residues can accumulate in the soil and food chain. Therefore, the application of many agrochemicals was banned or restricted, because of the environmental and health risks to humankind and soil fertility, and (2) resistance of genetically resistant cultivars is often broken by the pathogen within a few years and frequently accompanied by a reduction in yield. Typically, there is a lack of acceptance among the public for genetically modified (GM) crops.

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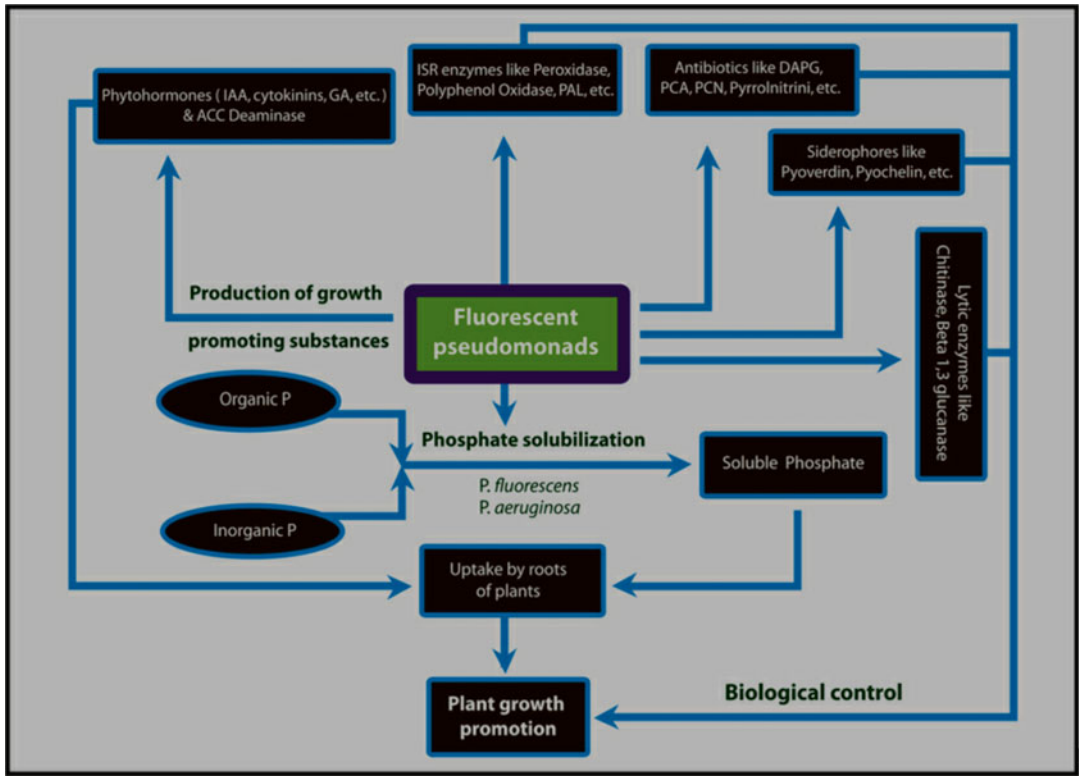
Phytohormones (IAA, cytokinins, GA, etc.) & ACC Deaminase
ISR enzymes like Peroxidase, Polyphenol Oxidase, PAL, etc.
Antibiotics like DAPG, PCA, PCN, Pyrrolnitrini, etc.
Siderophores like Pyoverdin, Pyochelin, etc.
Lytic enzymes like Chitinase, Beta 1,3 glucanase
Production of growth promoting substances
Fluorescent pseudomonads
Organic P
Inorganic P
Phosphate solubilization
P. fluorescens
P. aeruginosa
Soluble Phosphate
Uptake by roots of plants
Plant growth promotion
Biological control

Fig. 9.1 Direct and indirect mechanisms involved in plant growth promotion by fluorescent pseudomonads (Adapted and modified from Khan et al. (2010))

IDM, which combines biological, cultural, physical, and chemical control strategies in a holistic way rather than using a single component strategy, proved to be more effective and sustainable. In practice and in the majority of cropping systems today, emphasis is still being placed on a single technology. The concept of IDM, where diseases are managed by integrating a range of control methods and practices, is becoming more popular among farmers and researchers. The success and sustainability of IDM strategy, especially with resource-poor farmers, greatly depend on their involvement in helping generate locally specific techniques and solutions suitable for their particular farming systems and integrating control components that are ecologically sound and readily available to them (Khoury and Makkouk 2010). Biological control of plant diseases has been considered a viable alternative method to manage diseases. Biological control is the inhibition of growth, infection, or reproduction of one organism using another organism. Biocontrol is environmentally safe and in some case studies is the only option available to protect plants against pathogens (Heydari and Pessarakli 2010). Fravel (2005) reported 14 bacteria and 12 fungi registered with the United States Environmental Protection Agency for the control of plant diseases. Among the 14 registered bacterial biocontrol agents, six are based on *Bacillus*, five on *Pseudomonas*, two on *Agrobacterium*, and one on *Streptomyces*.

Pseudomonas spp. are particularly suited as biocontrol agents because they can use diverse root exudates as nutrient source; they are abundantly present in natural soils, especially in the rhizosphere; they have a high growth rate; they can be directly plant growth promoting; and they have the ability to control diseases by a variety of mechanisms as shown in Fig. 9.1. They are also the most extensively studied group of bacterial biocontrol agents, since *Pseudomonas* bacteria

are amenable to mutation and modification using genetic tools (Hofte and Altier 2010).

The chapter summarizes and discusses various studies of pseudomonads from the plant rhizosphere and their use for exploring disease management in IDM for sustainable agriculture.

Ecological Distribution of Pseudomonads in Plants

Pseudomonas is one of the most ubiquitous bacterial genera in the world, and different species have been isolated from very diverse ecological niches. Only the representatives of rRNA group I were included in the genus *Pseudomonas*, and up to now, 128 species have been validly described for this genus, including the fluorescent pseudomonads that have the capacity to produce fluorescent pyoverdine-type siderophores under low-iron conditions. Most species are saprophytes that are commonly found in water and soil; 23 species are pathogenic to plants, including *P. syringae* with 36 pathovars affecting different plants. In addition, 16 species are associated with diseases in humans and animals (Peix et al. 2009). Some species, such as *P. aeruginosa*, are ubiquitous and can be associated with both plants and animals. Some of the saprophytic species have interesting characteristics and are used in biotechnological applications to improve plant growth and plant health, but also in water and soil bioremediation. *P. fluorescens* biovar III has been reported as the dominant group of bacteria among fluorescent pseudomonads associated with the rhizosphere of rice, the most important food crop of the world. *P. fluorescens* biovar V constituted more than 75 % of fluorescent pseudomonads in at least some areas of the world and represented as the predominant group in Australian soils and wheat rhizosphere. Nearly 90 % of fluorescent pseudomonads in Colombia were reported to be *P. putida*. Thus, vast microbial diversity occurs among fluorescent pseudomonads in different geographic origins, and this group of bacteria exhibits an array of mechanisms that mediate biocontrol of pathogens and growth promotion of crops (Pathama et al. 2011).

Pseudomonas spp. diversity is also found in many plant rhizosphere and phyllosphere environments (van den Broek et al. 2005; Hirano and Upper 2000). Many researchers reported culture-independent analysis for examination of *Pseudomonas* assemblage in the endosphere by using genus-specific primers (Garbeva et al. 2001; Reiter et al. 2002; Sessitsch et al. 2002). Root tissue analysis indicated that the dominant pseudomonad species in flax was *P. putida* and in tomato, *P. fluorescens*. Fluorescent *Pseudomonas* were reported as competitive in the rhizoplane of maize, as there was an increase in the heterotrophic and total gram-negative communities present inside the rhizoplane. Thirty percent of isolates were identified as *P. fluorescens* and 50 % were identified as *P. putida* (Chaudhary et al. 2009).

Pseudomonads as Potential PGPR by Direct Mechanisms Under Field Conditions

Rhizosphere microorganisms may provide a front line defense against pathogen attack and are ideal for use as biocontrol agents. Some bacteria are associated with roots of crop plants and exert beneficial effects on their hosts and are referred to as plant growth-promoting rhizobacteria (PGPR) (Lugtenberg and Kamilova 2009). PGPR are free-living soil bacteria that can either directly or indirectly facilitate rooting and growth of plants. Direct mechanisms include nitrogen fixation, siderophore production, phosphate solubilization, and synthesis of phytohormones. PGPR indirectly enhance plant growth via suppression of phytopathogens by a variety of mechanisms. These include the ability to produce siderophores that chelate iron, making it unavailable to pathogens; the ability to synthesize antifungal metabolites such as antibiotics, fungal cell wall-lysing enzymes, or production of volatiles such as hydrogen cyanide, which suppresses the growth of fungal pathogens; the ability to successfully compete with pathogens for nutrients or specific niches on the root; and the ability to induce systemic resistance (ISR) (Saraf et al. 2010). Zabihi et al. (2011) studied that *P. putida* 108 was the most

effective strain-enhancing wheat P uptake and grain yield under greenhouse (96 and 58 %) and field (80 and 37 %) conditions, respectively. Hence, although *Pseudomonas* sp. could be a suitable replacement for high P fertilization, however, the optimum wheat yield resulted when the bioinoculants are combined with 50 % (25 kg/ha P_2O_5) P fertilization. This finding has great agricultural and environmental implications. The presence of *P. fluorescens* inoculants in the combination of microbial fertilizer plays an effective role in stimulating the yield and growth traits of chickpea (Rokhzadi et al. 2008). Five *P. putida*, one *P. graminis*, and one *P. fluorescens* strains showed significant increases in fresh and dry masses of sugarcane plantlets in tissue culture under gnotobiotic conditions (Mehnaz et al. 2009). Husen et al. (2011) evaluated the effects of eight promising isolates of ACC deaminase-producing pseudomonads on soybean growth and yield under field soil conditions. Field performance of five *P. fluorescens* isolates (Pf-173, Pf-193, Pf-547, Pf-551, and Pf-572) for biocontrol and plant growth-promoting activities were evaluated in French bean (var. Pusa contender). Germinated French bean seedlings exhibited enhanced root and shoot lengths in all the treatments. The combination of the five isolates proved to be the best, with maximum disease reduction (93.33 %) and enhancement of various plant growth parameters. Among individual isolates, Pf-551 had the best performance for most of the parameters assessed (Negi et al. 2011). Wahyudi et al. (2011) examined 14 isolates from the soybean rhizosphere as pseudomonads spp. that showed phosphate-solubilizing activity, siderophore production, IAA production, and promoted enhancement of root length, shoot length, or a number of lateral roots of soybean. Plant growth attributes produced by fluorescent pseudomonads that promote plant growth are listed in Table 9.1.

Naz and Bano (2010) reported three species of *Pseudomonas*, viz., *P. mendocina* Khsr2, *P. stutzeri* Khsr3, and *P. putida* Khsr4, for their phosphate solubilization and phytohormone production (indole-3-acetic acid, gibberellic acid, *trans*-zeatin riboside, and abscisic acid) in culture media and were found to be efficient in stimulating root/shoot length and dry weight and proline contents of *Zea mays* L. (advance germplasm line: Islamabad Gold) seedlings grown under normal and NaCl stress (20 dS m^{-1}). The strain *P. stutzeri* Khsr3 appears to be a potential candidate as a bioinoculant for saline fields. Mishra et al. (2011) observed that inoculation with *Pseudomonas* sp. strain PGERs17 exhibits more vigorous vegetative growth with increase in nodulation (55.0 %), leghaemoglobin content (27.7 %), physiologically available iron (24.1 %), total iron (90.7 %), chlorophyll content (43.9 %), P uptake (37.1 %), and N uptake (27.1 %) over mono-inoculation of *R. leguminosarum*-PR1 60 days after sowing (DAS) of lentil.

Pseudomonads as Biocontrol Agents Against Soil borne Pathogens

Pseudomonads are effective against a number of Soil borne pathogens through their both direct and indirect mechanisms. Antibiosis involving secretion of antibiotics, siderophore, and fungal cell wall-lysing enzymes is a direct mechanism of biocontrol, whereas induction of disease resistance in plants is an indirect mechanism of plant growth promotion.

Antibiosis

Phloroglucinol

Phloroglucinols are phenolic compounds by nature and bacterial and plant metabolites with broad-spectrum antiviral, antibacterial, antifungal, antihelminthic, and phytotoxic properties. The compound 2,4-diacetylphloroglucinol (2,4-DAPG), which is produced by certain plant-associated fluorescent *Pseudomonas* species, is of particular significance to agriculture because of its activity against a variety of root and seedling-borne pathogens of plants (Pathama et al. 2011).

Phenazines

Phenazines (Phz) are N-containing heterocyclic pigments produced by various pseudomonad species. Phz also play an important role of microbial

Table 9.1 Plant growth-promoting characteristics of fluorescent pseudomonads

Pigments, enzymes, and hormones	*Pseudomonas* species	References
Siderophore		
Pyoverdine	*Pseudomonas* sp.	Tank and Saraf (2009)
	P. fluorescens 3551	Loper (1988)
	P. fluorescens CHAO	
Pyochelin	*P. putida* WCS358	Maurhofer et al. (1994)
	P. aeruginosa 7NSK2	
Pseudomonine	*P. stutzeri*	Van Wees et al. (1977)
	P. fluorescens WCS374	Buysens et al. (1996)
	P. aeruginosa NJ-15	Lewis et al. (2000)
	P. monteilii FPB59, FPB63	Mercado-Blanco et al. (2001)
	P. plecoglossicida FPB31	Bano and Musarrat (2003)
		Ravindra Naik et al. (2008)
		Ravindra Naik et al. (2008)
Phosphate		
Solubilization	*P. mosselii* FP13	Jha et al. (2009)
Phosphatase	*P. fluorescens* CHAO	De Werra et al. (2009)
	P. putida MSC1 and *P. pseudoalcaligens* MSC4	Patel et al. (2011)
Denitrifying enzymes	*P. putida* GR 12-2	Patten and Glick (2002)
	P. mosselii FP13	Ravindra Naik et al. (2008)
	P. aeruginosa FP 10	Ayyadurai et al. (2006)
Indole-3-acetic acid	*P. fluorescens* Psd	Upadhyay and Srivastava (2010)
	P. aeruginosa PS1	Ahemad and Khan (2011a)
	P. putida PS9	Ahemad and Khan (2011b)
ACC deaminase	*P. fluorescens*	Shaharoona et al. (2008)
	P. jessenii	Rajkumar and Freitas (2008)
	P. aeruginosa and *P. fluorescens*	Tank and Saraf (2010)

competition in rhizosphere, including survival and competence. Phenazine-1-carboxylic acid (PCA) has been reported from fluorescent pseudomonads such as *P. fluorescens*, *P. chlororaphis*, and *P. aeruginosa* (Quan et al. 2010).

Pyrrolnitrin

Pyrrolnitrin (PRN) (3-chloro-4-(20-nitro-30-chlorophenyl) pyrrole) has a broad-spectrum antifungal activity as first described by Arima et al. (1964). PRN is a secondary metabolite derived from L-tryptophan during metabolism. It is produced by fluorescent pseudomonads such as *P. fluorescens* (Kirner et al. 1998) and *P. aureofaciens* (Elander et al. 1968). Other variants of PRN such as isopyrrolnitrin, oxypyrrolnitrin, and monodichloropyrrolnitrin have lower antifungal activity. PRN is active against a wide range of deuteromycete, ascomycete, and basidiomycete fungi.

Cyclic Lipopeptides (CLP)

Production of different kinds of CLP is common among fluorescent *Pseudomonas* spp. All CLPs have either 9 or 11 amino acids in the peptide ring with a C10 fatty acid attached at one of the amino acids (Nielsen et al. 2002). Its synthesis is non-ribosomal and catalyzed by large peptide synthetase complexes (Marahiel et al. 1997). CLP is involved in the promotion of bacterial swarming (Givskov et al. 1998; Lindum et al. 1998), with antimicrobial (Takesako et al. 1993; Gerard et al. 1997; Vollenbroich et al. 1997) and biosurfactant properties (Rosenberg and Ron 1999). Strains of *P. fluorescens*, DR54, 96.578,

and DSS73, produce three different CLPs, viz., viscosinamide (Nielsen et al. 2002), tensin (Henriksen et al. 2000), and amphisin (Sorensen et al. 2001). Apart from the antifungal action of viscosinamide, it is also involved in the primary metabolism and cell proliferation and strongly binds to the producing cells of the strain DR54 (Nielsen et al. 1999). Tensin and amphisin produced are released into the surrounding medium and suppress the ingress of the pathogen (Nielsen et al. 2000). Amphisin is a new member of a group of dual-functioning compounds like tensin, viscosin, and viscosinamide that have both biosurfactant and antifungal properties.

Amphisin is produced during the stationary phase. *amsY* gene codes for the synthesis of amphisin synthetase, controlled by a two-component regulatory system GacA/GacS (Koch et al. 2002). The ability of *P. fluorescens* strain DSS73 to control *P. ultimum* and *R. solani* arises from amphisin-dependent surface translocation and growth by which the bacterium inhibits *P. ultimum* and *R. solani* (Andersen et al. 2003). The synergistic effect of surface motility and the synthesis of antifungal compounds could efficiently check and terminate the growth of pathogen and prevent the plants from infection by the pathogens. Massetolides are cyclic lipopeptide antibiotics produced by various *Pseudomonas* strains. Three genes were found to be involved in the massetolide A biosynthesis in *P. fluorescens* strain SS101. Massetolide A production is essential in the swarming motility of *P. fluorescens* SS101 and plays an important role in biofilm formation (de Bruijn et al. 2008).

Siderophore: Competition for Iron

Pyoverdine

Pyoverdine has a great affinity for ferric iron than the pyochelin. These compounds are water-soluble pigments that fluoresce yellow green under ultraviolet light. They all are structurally similar being comprised of a chromophore moiety, which confers on the bacterium its fluorescent nature, to which is bound a peptide chain of variable composition and a dicarboxylic acid (Glick et al. 1999).

Pyochelin

When plant-associated pseudomonads such as biocontrol strains of *P. fluorescens* and *P. putida* are deprived of iron, they produce two different types of siderophore: one is pyochelins and pyoverdines. Pyochelins are small, phenolate siderophores derived from salicylic acid and cysteine. Two pyochelin molecules bind with one molecule of ferric iron with a relatively low efficiency (Cox et al. 1981; Crosa 1989).

Salicylic Acid

Some *Pseudomonas* spp. can produce a nonfluorescent siderophore called pyochelin, consisting of a salicylic-substituted cysteinyl peptide. Salicylic acid is considered to be not only an intermediate in pyochelin biosynthesis but also an endogenous siderophore for some *Pseudomonas* spp. Salicylic acid is produced under iron limitation conditions (Leeman et al. 1996).

HCN Production

HCN is an effective inhibitor of cytochrome c oxidase (Knowles 1976) and other metalloenzymes. Its production by PGPR is implicated in biological control of black root rot of tobacco (Voisard et al. 1989), root rot of tomato, and *Pythium* damping-off of cucumbers (Ramette et al. 2003). Cyanogenesis by *Pseudomonas* spp. is the product of an HCN synthase, encoded by three biosynthetic genes (hcnABC) (Laville et al. 1998). These three genes are important for HCN biosynthesis from glycine, the primary precursor (Blumer and Haas 2000).

Fungal Cell Wall-Degrading Enzymes

Antagonistic effects on phytopathogens can also be mediated by extracellular lytic enzymes, and many biocontrol pseudomonads produce such enzymes like chitinase, β-1,3-glucanase, protease, and cellulase (Chernin and Chet 2002). Chitinolysis plays an important role in the biological control of plant diseases and has been substantiated with increased disease control by

chitin-supplemented application of chitinolytic biocontrol agents. Chitinase and β-1,3-glucanase are the key enzymes associated with the decomposition of the fungal cell wall of Soil borne fungal pathogens. It has also been demonstrated that extracellular chitinase and laminarinase synthesized by *P. stutzeri* digest and lyse mycelium of *Fusarium solani* (Kumar et al. 2010). Chitinase and cellulase produced by pseudomonad strains showed mycelial growth inhibition of different fungal pathogens on PDA medium (Sindhu and Dadarwal 2001). Production of extracellular β-1,3-glucanases, chitinases, and proteinase increases significantly when *Pseudomonas* spp. are grown in media supplemented with either autoclaved mycelium or isolated purified host fungal cell walls (Viswanathan and Samiyappan 2000). Such induction of chitinases and their antifungal activity together with the fact that chitin, β-1,3-glucan, and protein are the main structural components of most fungal cell walls suggested that hydrolytic enzymes produced by some fluorescent pseudomonads play an important role in destruction of plant pathogens. Saraf et al. (2008) studied that *Pseudomonas* spp. (M1P3) showed maximum chitinase activity (40 µg/ml N-acetyl-glucosamine production) and also induced maximum cellulase production. Arora et al. (2008) reported fluorescent *Pseudomonas* (PGC 1 and PGC 2) for their antifungal potential against *R. solani* and *P. capsici*. The results of this study indicated the role of chitinase and β -1,3-glucanase in the inhibition of *R. solani*; however, antifungal metabolites of non-enzymatic nature were responsible for inhibition of *P. capsici*. Aeron et al. (2011) reported that *P. fluorescens* PS1 caused morphological alteration in mycelia of *S. sclerotiorum* as evidenced by hyphal perforation and fragmented lysis by extracellular enzymes chitinase (0.69 units/ml) and β-1,3-glucanase (1.98 units/ml).

Induced Systemic Resistance

Colonization of the rhizosphere by certain bacteria such as nonpathogenic pseudomonads can trigger a systemic resistance in plants by activating the plants' defense mechanisms phenotypically similar to pathogen-induced systemic acquired resistance (SAR), and this form of induced resistance is referred as rhizobacteria-mediated ISR. Fluorescent pseudomonads are also involved in the induction of plant resistance against insects and nematodes, and specific metabolites produced by these bacteria have been involved in the elicitation of defense reactions of the host plants. Similar to classical induced resistance, PGPR-mediated resistance is known to produce broad-spectrum resistance, which is correlated with increased amounts of pathogenesis-related (PR) proteins, peroxidase, chitinases, and β-1,3-glucanase in plant tissue (Pathama et al. 2011). A salicylate-overproducing recombinant of *P. fluorescens* strain P3 causes enhanced protection to tobacco against tobacco necrosis virus compared with the wild-type P3, which indicates that salicylate might also stimulate defense. In another strain of *Pseudomonas*, a combination of pyocyanin and pyochelin seems to be most effective for inducing resistance in tomato (Audenaert et al. 2002). Rajkumar et al. (2008) studied fluorescent pseudomonads (SE21 and RD 41) and resistance inducers (chitin and salicylic acid) for plant growth promotion and biological control of damping-off of pepper caused by *Rhizoctonia solani. P. fluorescens* strains CHA0 and Pf1 were investigated for their biocontrol efficacy against banana bunchy top virus (BBTV) in banana (*Musa* spp.) alone and in combination with chitin under glasshouse and field conditions. Bioformulation of *P. fluorescens* strain CHA0 with chitin was effective in reducing the banana bunchy top disease (BBTD) incidence in banana under glasshouse and field conditions. In addition to disease control, the bioformulation increased the economic yield significantly compared to the untreated control. Increased accumulation of oxidative enzymes, peroxidase (PO), polyphenol oxidase (PPO), phenylalanine ammonia lyase (PAL), pathogenesis-related (PR) proteins, chitinase, β-1,3-glucanase, and phenolics was observed in CHA0 bioformulation amended with chitin-treated plants challenged with BBTV under glasshouse conditions (Kavino et al. 2008).

Biosynthesis of Antifungal Metabolites and Their Genome Sequence Features

There are many strains of pseudomonads reported for their biofertilizer and biocontrol potential, but only few strains are known for their whole genome sequences with their metabolic pathway. Three *P. fluorescens* strains (biocontrol agents SWB25 and Pf-5 and the soil inhabitant Pfo-1) were compared, and results showed that they share only 61 % of their genes. The study revealed that these strains are a species complex rather than a single species (Silby et al. 2009). The antibiotic-productive gene loci are highly conserved and most intensively studies found that they correlated with DAPG-producing strains (Keel et al. 1996; Frapolli et al. 2007). *P. fluorescens* F113 complete genome sequence contained a large number of unique protein-coding genes that encode traits potentially involved in the rhizocompetence of this strain, and the features include protein-coding genes required for denitrification, diterpenoid catabolism, motility and chemotaxis, protein secretion, and production of antimicrobial compounds and insect toxins (Renodo-Nieto et al. 2013).

Nowadays, scientists are concentrating on the biotechnology of *P. fluorescens* for effective application on agricultural crops (Wiyono et al. 2008). Loper et al. (2007) reported that *P. fluorescens* Pf-5 produced three novel metabolite synthesis gene clusters that were identified in the genome attribute to biological control mechanisms. In general practices, sequenced strains present only a fraction of pseudomonad species diversity, and much of the group's metabolic and genetic diversity remains unexplored till today. Comparative genome analyses of ten strains within the *Pseudomonas fluorescens* group including seven new genome sequences were reported by Loper et al. (2012). They discovered genes for traits that were not known previously, including genes for the biosynthesis of the siderophores achromobactin and pseudomonine and the antibiotic 2-hexyl-5-propyl-alkylresorcinol; novel bacteriocins; type II, III, and VI secretion systems; and insect toxins. The genome of the nitrogen-fixing bacterium *Azotobacter vinelandii*, classified in the *Pseudomonadaceae*, has also been sequenced (Setubal et al. 2009). Cyanogenesis by *Pseudomonas* spp. is the product of an HCN synthase, encoded by three biosynthetic genes (hcnABC) (Laville et al. 1998). These three genes are sufficient for HCN biosynthesis from glycine, the primary precursor (Blumer and Haas 2000). Conservation of *phl* genes for biosynthesis of DAPG among ecologically and geographically diverse antagonistic pseudomonads further supports the global importance of DAPG production in biocontrol (Duffy et al. 2004). Biosynthesis of allelochemicals by *Pseudomonas* biocontrol potential strains is closely regulated by molecules produced by the organism itself and by external environmental factors such as nutritional components, soil chemical and physical properties, host plant genotype, and nonpathogenic soil bacteria, because these factors can determine the ability of particular strains to suppress disease. Identification and characterization at molecular levels would facilitate the targeted application of strains into environments that are more favorable for effective and consistent biocontrol activity (Saraf et al. 2014). Four biosynthetic genes (prnABCD) are highly conserved among strains of *P. fluorescens* that produce pyrrolnitrin (Hammer et al. 1997, 1999; Kirner et al. 1998). Expression of prnABCD in heterologous bacteria resulted in pyrrolnitrin production. Results concluded that these genes are sufficient for biosynthesis of the antibiotics from primary precursors (Hammer et al. 1997).

Role of Antifungal Metabolites in Disease Management Under Field Conditions

Indigenous DAPG-producing populations have been identified as the driving force behind the development of natural disease suppressiveness in certain soils under long-term monoculture. Meynet et al. (2011) reported that DAPG-producing *P. fluorescens* F113 enhanced *Azospirillum brasilense* Sp245-Rif traits involved in root colonization (cell motility, biofilm formation, and poly-β-hydroxybutyrate production) and phytostimulation (auxin production) of wheat. Pyoluteorin-producing bacteria *P. putida* strain NH-50 significantly reduced disease severity on both sugarcane varieties, irrespective of fungal inoculation, i.e., either inoculated through stem or

Table 9.2 Commercially available biocontrol products

Commercial product	Name of strain	Crop	Manufacturer
Bio-Save 10, 11, 100, 110, 1000™	*P. syringae ESC-100*	Pome fruit (Bio-Save 100) and citrus (Bio-Save 1000)	Eco Science Corp, Produce Systems Div., Orlando
Blight Ban A506	*P. fluorescens* A 506	Almond, apple, apricot, blueberry, cherry, peach, pear, potato, strawberry, tomato	Plant Health Technologies, USA
Conquer™	*P. fluorescens*	Mushrooms	Mauri Foods, Australia
Victus™	*P. fluorescens*	Mushrooms	Mauri Foods, Australia
Biomonas	*P. fluorescens*	Cotton, cereals, pulses, vegetables, oilseeds, fruit plants, and floriculture	Biotech International Limited, New Delhi
BIOWIN	*P. fluorescens*	Paddy, turmeric, banana, sugarcane, spices, oilseeds, pulses, cotton, and vegetables	Esvin Advanced Technologies Limited, Tamil Nadu
Phasal Rakshak powder	*P. fluorescens* 1 % W.P.	Wheat, chickpea, pigeon-pea, paddy, groundnut, mustard, sunflower, rapeseed, vegetables, fruits, citrus, apple, mango and all other crops	International Panaacea Limited, New Delhi
Bactvipe liquid	*P. fluorescens* 2 % A.S.		

Modified from Nakkeeran et al. (2005)

through soil. This strain also possessed other plant growth characteristics and can be used as a biopesticide for sugarcane crop (Hassan et al. 2011).

Siddiqui et al. (2003) have clearly proved the protective nature of HCN produced by *P. aeruginosa* against *Meloidogyne javanica*, the root-knot nematode of tomato. Siderophores such as pyoverdine and pyochelin have been documented well for the suppression of *Pythium*-induced damping-off disease of tomato (Buysens et al. 1996). Recently, Shen et al. (2014) reported that 100-fold dilution of *P. fluorescens* CZ powder could be used as application under greenhouse and field conditions which inhibited virus infection by 59.2 % on *N. tabacum* cv. NC89 plants in the greenhouse. In field trials, 100-fold dilution of CZ suppressed TMV infection by 58.2 % and 47.6 % in 2010 and 2011, respectively. D-gluconic acid was the most significant antifungal agent produced by *Pseudomonas* spp. strain AN5 for the biocontrol of take all of wheat roots (Kaur et al. 2006). These results interpreted that diverse compounds can be associated for disease suppression. Botelho and Selecaode (2001) concluded that phenazine production was associated with the "sudden death syndrome" caused by *F. solani* infecting soybean. Simultaneous production of DAPG/HCN from *Pseudomonas* sp. LBUM300 showed great potential for controlling the bacterial canker of tomato caused by *Clavibacter michiganensis* (Lanteigne et al. 2012). *Pseudomonas* strain GRP3 is known to produce rhamnolipids and siderophore and has been shown to reduce the occurrence of pre- and postemergence damping-off in chili and tomato (Sharma et al. 2007). Le et al. (2011) studied that phenazine- and lipopeptide-producing *Pseudomonas* spp. inhibited the hyphal growth of *S. rolfsii* and significantly reduced the stem rot disease of groundnut in greenhouse and field experiments. Kishore et al. (2006) also found that the biocontrol agent *P. aeruginosa* GSE 18 reduced the preemergence of groundnut rotting by 60 % in *A. niger*-infested potting mixture. Bhatia et al. (2008) reported increased seed germination, growth promotion, and suppression of charcoal rot due to *M. phaseolina* with fluorescent pseudomonads. Table 9.2 lists some of the commercially available biocontrol products obtained from pseudomonads in the market around the globe. The products are based on the success of pseudomonads as biocontrol agents because of their diverse mechanisms working under variable conditions and for different phytopathogens.

Conclusion

*Pseudomonas s*pp. are prolific producers of many secondary metabolites, and the availability of the genome features for several strains now opens new insight for the discovery of novel natural products for commercialization. The forthcoming developments by functional genomics approach using sequencing and proteome technology will help us to understand the performance of biocontrol strains from fluorescent and nonfluorescent pseudomonad species and overcome the current bottlenecks restricting their commercial use. The major challenges to be resolved prior to widespread commercial exploitation of biocontrol strains lie in the ability to predict more confidently the behavior of such strains in the field. Fortunately, as interest in these organisms grows, more information on the genetics, physiology, and ecology of metabolite production is becoming available. Such data are of immense importance for the selection of wild-type strains with desirable traits from nature and to provide a more rational framework for the choice of strains for use in microbial consortial inoculants. Recombinant DNA methods have enabled genetic manipulation of metabolite production with promising results. The use of metagenomics in parallel with established or novel molecular approaches such as genome mining will undoubtedly lead to a fresh look on the biology and practical applications.

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Pseudomonads: Plant Growth Promotion and Beyond

10

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Abstract

Members of the genus *Pseudomonas* are a ubiquitous and important component of the soil and rhizospheric ecosystems, where they play multifarious roles such as the recycling of organic matter, promotion of plant growth, alleviation of abiotic stress effects in plants, and degradation of xenobiotic compounds. The versatility and ecological fitness of this genus has been often attributed to its metabolic versatility and its ability to produce antagonistic molecules, thereby gaining a niche advantage in the rhizosphere. This chapter attempts to briefly explore the historical evolution of this genus and explore the various facets of Pseudomonads, which confer upon them a distinct edge over other soil microbes. The utility of Pseudomonads both within and beyond the realm of plant growth promotion, in the agro and related ecosystems, is also discussed in brief.

Introduction

The genus *Pseudomonas* (derived from the Greek words *pseudes* "false" and *monas* "a single unit" or "false unit") comprises one of the most diverse and ecologically fit groups of bacteria on this planet, whose members are collectively referred to by the generic term Pseudomonads. Though at times the term "Pseudomonad" is also used to refer to former members of the genus *Pseudomonas* that have been reclassified into other genera at present, for greater clarity sake we shall be limiting our usage of this term to denote only the existing members of the genus *Pseudomonas*. Taxonomically the genus *Pseudomonas* falls within the γ subclass of Proteobacteria, and its ubiquity can be gauged by its ability to colonize terrestrial, freshwater, and marine environments with relative ease, besides forming intimate associations with higher forms of life. But its marked preference for plants in forming associations is well known. Pseudomonads are able to utilize an array of organic compounds as energy sources (Lessie and Phibbs 1984) and produce diverse secondary metabolites (Raaijmakers et al. 2002; Gross and Loper 2009). Universal distribution and remarkable degrees of physiological adaptations make them one of the most sought after and studied bacterial species.

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In the agro-ecosystem, Pseudomonads play roles as diverse as the decomposition of organic matter, promotion of plant growth, protection of plants from diseases, control of insect pests, and nutrient solubilization. Other associated roles that have been attributed to Pseudomonads include their ability to metabolize a variety of xenobiotic compounds (Pak et al. 2000) and the alleviation of abiotic stress effects on plants (Mishra et al. 2011). The genus *Pseudomonas* comprises of more than 100 named species that are divided into lineages, groups, and subgroups based on multilocus sequence analysis (Yamamoto et al. 2000; Guttman et al. 2008; Mulet et al. 2010). The *Pseudomonas fluorescens* group alone encompasses more than 50 named species of plant-associated commensals and has therefore emerged as an agriculturally important group among the Pseudomonads. Plant-associated roles of the genus *Pseudomonas* vary from being the causative agents of several diseases to abiotic stress alleviation in plants. Therefore, considering their ubiquity and metabolic and functional versatility, it can be rightly said that Pseudomonads are the bacterial species of prime importance in the agro-ecosystem. This chapter attempts to trace the reasons for Pseudomonad diversity and their utility in the agro-ecosystem, within and beyond the realm of plant growth promotion.

Pseudomonads: A Historical Perspective

The genus *Pseudomonas* was first described by Migula (1894) and has been a part of the history of bacteriology ever since its inception. Though initially *Pseudomonas* was described as a genus of Gram-negative, rod-shaped, and polar flagellated bacteria with some sporulating species, it was later proved that the so-called spores were actually refractive granules of reserve materials. The physiological diversity of this bacterial genus was unraveled by the pioneering studies of bacteriologists such as Beijerinck (1921) and Winogradsky (1949) during the first half of the twentieth century. The 1923 edition of the Bergey's Manual (Bergey et al. 1923) included a chapter on the genus *Pseudomonas*, where the classification of species was based on phenotypic characteristics. But the initial classification of *Pseudomonas* as Gram-negative, aerobic, non-sporulated rods that are motile by means of polar flagella did not differentiate it sufficiently from other Gram-negative bacteria and led to the dumping of several improperly characterized Gram-negative bacteria. But latter-day observations that incorporated other phenotypic characteristics were able to bring about a degree of clarity on the taxonomical boundaries of this genus. A monumental work in the 1960s, to clarify the taxonomy of the genus, was carried out by Stanier et al. (1966). This exhaustive and highly cited work which reported the nutritional characteristics of 267 strains on 146 different organic compounds plus a wide range of associated characteristics is considered as a classic study on bacterial taxonomy (Spiers et al. 2000). The development of DNA-DNA hybridization methodologies, during the same decade, led to the confirmation and reclassification of several phenotypic data, which ultimately led to the inclusion of the G+C content of bacteria in the Bergey's Manual edition published in 1974, which included 29 species (Doudoroff and Palleroni 1974). During this period another development in the classification of genus *Pseudomonas* by genotypic criteria was made by Palleroni and coworkers, who classified this bacterial group into five rRNA subgroups based on the measurements of RNA-DNA relatedness (Palleroni et al. 1973). These rRNA subdivisions were phylogenetically very distant, and only the representatives included in the rRNA group I were finally included in the genus *Pseudomonas*, while the members of the other subgroups were reclassified as separate genera. Though this approach failed to find a place in the 1974 edition of the Bergey's Manual, it was accommodated in the 1984 edition of the Bergey's Manual, with minor modifications in the number of species (Palleroni 1984).

The 1980s saw the emergence of bacterial taxonomy based on the sequencing of the 16S rRNA gene (Woese et al. 1984). But this scheme of

classification was not reflected in the Bergey's Manual of Determinative Bacteriology published in 1994, where species determination was carried solely on the basis of phenotypic characters. This edition included only the classical species of *Pseudomonas*, while the newly described species were included as an additional list. The current edition of Bergey's Manual of Systematic Bacteriology contains an exhaustive review of the criteria used in the modern taxonomy of genus *Pseudomonas* and includes 59 species that were described till the year 2005 (Palleroni 2005). Since then several new species of genus *Pseudomonas* have been described, and their number is continuously increasing. Other novel approaches used in the *Pseudomonas* taxonomy include siderotyping of both fluorescent and nonfluorescent Pseudomonads (Meyer et al. 2002) and fluorescent spectroscopy fingerprinting, by which Tourkya and collaborators were able to discriminate *Pseudomonas* at the genus level from *Burkholderia*, *Xanthomonas*, and *Stenotrophomonas*, with very high sensitivity. At the species level they were able to group *P. chlororaphis*, *P. lundensis*, *P. fragi*, *P. taetrolens*, and *P. stutzeri* separately from *P. putida*, *P. pseudoalcaligenes*, and *P. fluorescens* (Tourkya et al. 2009). It is obvious that ever since its discovery, the genus *Pseudomonas* has comprised a large number of species that have increased in magnitude over time often reaching alarming proportions (Palleroni 2008). But constant developments in bacterial taxonomy have led to the realignments at the generic level, and the genus *Pseudomonas* still retains its identity as a versatile and ubiquitous genus. A list of novel species of Pseudomonads from agro-environments that have been validly published subsequent to the year 2005 is listed in Table 10.1.

Table 10.1 Validly published *Pseudomonas* species originating from the agro-environment since the year 2005

Pseudomonas species	Origin	Reference
P. chengduensis	Landfill leachate	Tao et al. (2014)
P. kunmingensis	Phosphate mine	Xie et al. (2014)
P. punonensis	Straw	Ramos et al. (2013)
P. formosensis	Food-waste compost	Lin et al. (2013)
P. guariconensis	Rhizospheric soil	Toro et al. (2013)
P. sagittaria	Oil-contaminated soil	Lin et al. (2013)
P. zeshuii	Herbicide-contaminated soil	Feng et al. (2012)
P. baetica	Wedge sole	López et al. (2012)
P. bauzanensis	Soil	Zhang et al. (2011)
P. taiwanensis	Soil	Wang et al. (2010)
P. cedrina	Phyllosphere of grasses	Behrendt et al. (2009)
P. brassicacearum subsp. *Neoaurantiaca*	Soil and the rhizosphere of agricultural plants	Ivanova et al. (2009)
P. panipatensis	Oil-contaminated soil	Gupta et al. (2008)
P. lurida	Phyllosphere of grasses	Behrendt et al. (2007)
P. reinekei	Soil	Cámara et al. (2007)
P. moorei	Soil	Cámara et al. (2007)
P. mohnii	Soil	Cámara et al. (2007)
P. moraviensis	Soil	Tvrzová et al. (2006)
P. segetis	Soil	Tvrzová et al. (2006)
P. panacis	Soil	Park et al. (2006)

Diversity of Pseudomonads

Diversity in living organisms occurs as a result of interplay between ecological and genetic factors. The ultimate cause of diversity is mutation, but the variations generated by mutations and recombination are subject to selection pressures and genetic drift (Bell 1997; Rainey et al. 2000).

Pseudomonads are one of the most diverse groups of bacteria on this planet and comprise an array of environmental and clinical species. This genus includes the well-known plant-pathogenic bacterium *Pseudomonas syringae* and the opportunistic human pathogen *Pseudomonas aeruginosa*.

Apart from these, several environmental species that are either beneficial or neutral in their interaction with plants and several opportunistic human/veterinary pathogens are also found. Pseudomonads occur in environments as diverse as distilled water (Favero et al. 1971), ocean water (Khan et al. 2007), dairy products (Dogan and Boor 2003), meat (Ayres 1960), internal tissues of plants (Zinniel et al. 2002), animal tissues (Ramphal et al. 1980), domestic and hospital areas (Whitby and Rampling 1972). Environmental isolates of *Pseudomonas* have been isolated from hot deserts (Bhatnagar and Bhatnagar 2005), cold alpine environments (Meyer et al. 2004), Antarctic soils (Panicker et al. 2002), oil spill sites (Das and Mukherjee 2007), heavy metal-contaminated sites (Roane and Kellogg 1996), acid soils (De Boer and Kowalchuk 2001), and mine spoils (Piotrowska-Seget et al. 2005). This diversity of Pseudomonads is not entirely limited to its phenotype alone, but is also reflected at the genetic level wherein the architecture of the chromosome and extrachromosomal elements are known to contribute to the ecological fitness and diversity of this genus (Spiers et al. 2000). The phenotypic and chemotaxonomic heterogeneity of the genus is reflected in its fairly broad DNA guanine plus cytosine (G+C) content ranging from 58 to 70 mol % (Palleroni 1984). Thus the diversity of Pseudomonads can be rightly ascribed to its metabolic and genetic versatility, both of which confer a level of ecological fitness to this genus that is rarely seen among bacteria. We shall briefly discuss the ecological and genetic basis of diversity among Pseudomonads in the following section.

Ecological Causes of Pseudomonad Diversity

Charles Darwin proposed that "ecological opportunity" is an essential factor and the primary cause of diversity within an organism. This statement is all the more relevant for bacteria which have the remarkable capacity to exploit the smallest of opportunities. The classical observations of Gause, that two distinct test tube populations of *Paramecium* competing for the same niche could not coexist and therefore led to the extinction of one population (Gause 1934), were followed by the niche exclusion principle, which states that no two organisms with similar requirements and characteristics can occupy the same niche. This principle postulates that if an environment lacks spatial or temporal heterogeneity (i.e., is homogeneous) and affords just a single niche, therefore in principle, it will house just a single species (Hardin 1960). Therefore, it is obvious that in a homogenous environment, as a population evolves, the more successful species simply replaces the existing species. This theory found support in the laboratory studies of Rainey and Travisano (1998) who observed that isogenic populations of *P. fluorescens* when grown in identical environments except for the degree of ecological opportunity (spatial structure that was provided by shaking), diverse niche-specialist genotypes, emerged rapidly in the spatially heterogeneous environment but not in the spatially homogeneous environment. Pseudomonad diversity in nature can therefore partly be attributed to the ability of this genus to utilize the ecological opportunity.

Competition is commonly attributed to be the driving force, or engine, of diversification (Abrams 1987). Let us assume an environment where a single bacterial species is grown; as the density of population increases, growth becomes limited due to depletion of resources such as oxygen and nutrients. Therefore, the competition becomes intense, and natural selection would favor a mutant that can gain access to the limiting resources. Hence the most competent genotypes are those that occupy newer niches unlike the one occupied by the dominant genotype, since scarce resources will be most abundant in the newer niches. This selection would continue to favor mutants that show increasing degrees of niche specialization so that eventually there will be minimal niche overlap among genotypes. If one were to apply this principle of diversity to Pseudomonads, it could be concluded that the metabolic diversity among Pseudomonads could have arisen due to the intense competition within and among populations. Other possible ecological

causes of Pseudomonad diversity can be attributed to the increased productivity and lowered mortality rates in populations and increased resistance to parasitic agents in heterogeneous environments (Spiers et al. 2000).

Genetic Basis of Pseudomonad Diversity

Considering the ecological, metabolic, and biochemical diversity of this genus, it is quite obvious that diversity extends to the genome sequence level. The complete genomes of many species have now been sequenced, and it has been observed that only 25–35 % of the genomes of the sequenced isolates are composed of core genes that are shared by all members of the genus (Silby et al. 2011). The genomes of *Pseudomonas* spp. are composed of relatively stable core regions that are interspersed with regions that vary among the strains (Silby et al. 2009, 2011). Some regions that are unique to a specific strain are predicted to confer the distinctive characteristics. The combined repertoire of the core and variable regions of a genome reflects the ecological history of the strain and the various environments or selective pressures that it has encountered over evolutionary time (Loper et al. 2012).

The genomes of *Pseudomonas* are usually larger in size with an extensive gene complement. While in other bacterial species, genomes are smaller in pathogenic isolates compared to the environmental isolates, in *Pseudomonas* there seems to be no correlation between the genome size and the lifestyles (pathogenic or saprophytic). Plant-pathogenic strains of *P. syringae*, opportunistic human pathogens of *P. aeruginosa*, and the insect pathogen *P. entomophila* have genome sizes ranging from 5.9 to 6.9 Mb (Loper et al. 2012). This observation finds support in the fact that plant-pathogenic strains of *P. syringae* can exist epiphytically on plant surfaces (O'brien and Lindow 1989), while *P. aeruginosa* is a common environmental habitant (Das and Mukherjee 2007), which indicates that the pathogenic lifestyle is just another mode of existence, rather than the sole mode of survival. The large genome seems to be an adaptation to handle the range of environments that these bacteria encounter.

Mutations are thought to be the foremost reason for the evolution of novel genotypes among bacteria, and they are thought to occur with rapid frequency under stress conditions because of the SOS response and the reduced ability to deal with DNA-damaging free radicals generated by metabolism (Friedberg et al. 1995). But it is to be deciphered as to whether the genome of *Pseudomonas* is more prone to mutation. This argument finds support with the isolation of mutator strains (with elevated mutation rates) of *P. aeruginosa* containing defective mismatch repair genes (Oliver et al. 2000). Since *Pseudomonas* strains occupy diverse environments, it's quite obvious that they are more prone to stress conditions and hence may be more prone to diversity-generating mutations.

The acquisition of genes and accessory genetic elements (plasmids, transposons, integrons, and phages) by lateral gene transfer is a pervasive and important source of innovation for bacterial evolution (Ochman et al. 2000). This is especially intriguing since with the exception of *P. stutzeri*, others are not known to be naturally competent (Spiers et al. 2000). Population genetic studies in *Pseudomonas* have shown that recombination occurs, but this seems to be within an overall pervasive clonal framework as evidenced by the repeated sampling of identical clones from different environments often years apart (Haubold and Rainey 1996; Kiewitz and Tummler 2000). But accessory genetic elements play an important role in the lateral movement and recombination of external DNA. Large plasmids encoding the entire catabolic pathways and associated regulatory genes are common in *Pseudomonas* (Sesma et al. 2000; Thomas 2000). Such plasmids often have host ranges that extend beyond the genus. An interesting observation by Selvakumar et al. (2009a), that the mountain aspect affects the genetic clustering of cold-tolerant Pseudomonads from high-altitude regions, has thrown another dimension to the ecological-genetic interaction determinants of diversity. They observed from

the rep-PCR profile that isolates originating from the cooler northern mountain aspects and warmer southern mountain aspects clustered separately and had distinct phosphate solubilization abilities. To conclude, it can be said that large genomes increased the frequencies of mutation and genetic recombination both at the chromosomal and extrachromosomal levels and play a major role in Pseudomonad diversity.

Plant Growth Promotion and Rhizospheric Competence of Pseudomonads

Pseudomonads are the most studied bacteria with reference to their ability to promote plant growth by a myriad of activities, and therefore a non-exhaustive compilation of plant growth promotion by Pseudomonads is listed in Table 10.2. Since several extensive reviews on plant growth promotion by Pseudomonads are available, we shall not dwell at length on the mechanisms of plant growth mechanism, but rather focus on the rhizospheric competence traits that confer upon *Pseudomonas* a pre-eminent position among rhizobacteria.

To achieve effective root colonization, the following traits, viz., motility (de Weger et al. 1987); production of the O-antigen of lipopolysaccharide (LPS) (de Weger et al. 1989); utilization of cellulose (Matthysse and McMahan 1998), thiamine (Simons et al. 1996), amino acids (Simons et al. 1997), and biotin (Streit et al. 1996); and the synthesis of an isoflavonoid-inducible efflux pump (Palumbo et al. 1998), are thought to be

Table 10.2 Plant growth promotion by different *Pseudomonas* species

Pseudomonas species	PGP effect/trait	Reference
Pseudomonas sp.	Seed piece inoculation caused a reduction of soft rot disease caused by *Erwinia carotovora*	Kloepper (1983)
P. fluorescens	Prevention of citrus canker caused by *Xanthomonas campestris* pv. *citri* by siderophore production	Unnamalai and Gnanamanickam (1984)
P. putida W4P63	Suppression of soft rot of *Erwinia carotovora* in tuber siderophore production	Xu and Gross (1986)
P. cepacia B5	Suppression of *R. solanacearum* by 2-ketogluconic acid	Aoki et al. (1991)
P. glumae	Suppression of *R. solanacearum* by induction of systemic resistance	Furuya et al. (1991)
P. fluorescens PF59	Suppression of *R. solanacearum* by siderophore production	Hartmann et al. (1992)
P. fluorescens A506	Reduction in populations of *Erwinia amylovora* in pear flowers due to competition	Wilson and Lindow (1993)
Pseudomonas sp.	Suppression of the tomato wilt pathogen by siderophore production	Jagadeesh et al. (2001)
P. fluorescens	Resists rice bacterial blight caused by *Xanthomonas oryzae* pv *oryzae* by induced systemic resistance (ISR)	Vidhyasekharan et al. (2001)
P. fluorescens PRS9 and *P. fluorescens* GRS1	Production of IAA, siderophores, and phosphate solubilization	Gupta et al. (2005)
Pseudomonas sp.	Phosphate solubilization, production of IAA and siderophores	Wani et al. (2007)
P. fluorescens	Induced systemic resistance and antifungal activity	Saravanakumar et al. (2007)
Pseudomonas sp.	Production of IAA, siderophores, antifungal activity, ammonia, and HCN production	Ahmad et al. (2008)

(continued)

Table 10.2 (continued)

Pseudomonas species	PGP effect/trait	Reference
P. tolaasii ACC23, *P. fluorescens* ACC9	Protects canola plants against the inhibitory effects of cadmium	Dell'Amico et al. (2008)
Pseudomonas spp.	Increased ability to control bacterial and fungal root pathogens of rice	Lawongsa et al. (2008)
Pseudomonas sp.	Cold-tolerant plant growth-promoting bacterium	Mishra et al. (2008)
Pseudomonas sp.	Production of ACC deaminase, IAA, and siderophore	Poonguzhali et al. (2008)
P. jessenii	Production of ACC deaminase, IAA, siderophore, heavy metal solubilization, and phosphate solubilization	Rajkumar and Freitas (2008)
P. aeruginosa	Stimulation of plant growth, reduced Cd uptake in Indian mustard and pumpkin	Sinha and Mukherjee (2008)
P. aeruginosa, *P. fluorescens*	Production of siderophores	Braud et al. (2009)
P. fragi	Solubilizes rock phosphate at low temperatures and promotes wheat seedling growth	Selvakumar et al. (2009a, b)
Pseudomonas sp. NARs9	Cold-tolerant plant growth-promoting bacterium	Mishra et al. (2009)
Pseudomonas sp. A3R3	Production of IAA and siderophores	Ma et al. (2011)
P. lurida	Cold-tolerant phosphate-solubilizing bacterium that promotes wheat seedling growth	Selvakumar et al. (2011)
Pseudomonas sp.	Significant increase in soil enzyme activities, total productivity and nutrient uptake in soybean and wheat	Sharma et al. (2011)
Pseudomonas sp.	Significant increase in plant dry weight, nodule numbers, total chlorophyll content, leghemoglobin, root N, shoot N, root P, shoot P, seed yield, and seed protein in green gram	Ahemad and Khan (2012a)
P. putida	IAA, siderophores, HCN, ammonia exo-polysaccharides, phosphate solubilization	Ahemad and Khan (2012b)
P. fluorescens SS101 (*Pf. SS101*)	Protects *Arabidopsis* from the infection of *P. syringae* pv *tomato* through induction of systemic resistance	Judith et al. (2012)
Pseudomonas sp.	Increases iron uptake in pea when co-inoculated with *Rhizobium*	Mishra et al. (2012)
P. brassicacearum J12	Suppresses *R. solanacearum* by 2,4-diacetylphloroglucinol (2,4-DAPG)	Zhou et al. (2012)
Pseudomonas spp.	Solubilizes rock phosphate at low temperatures and promotes lentil growth	Selvakumar et al. (2013)

essential, and mutants deficient in one or more of these traits have been proven to be poor root colonizers (Lugtenberg et al. 2001). Though motility confers a distinctive edge to an organism, it has been disputed by some workers (Howie et al. 1987). But it has been clearly proved that nonmotile mutants are clearly deficient in root colonization (Simons et al. 1996). Since *Pseudomonas* is known to produce up to nine flagella per cell, it is easy to screen for motile strains in laboratory media. It is further seen that chemotaxis-derived motility rather than random motility plays an important role in the root tip colonization (Lugtenberg et al. 2001). Hence chemotaxis and motility can serve as suitable traits for the selection of efficient root colonization strains. The pili or fimbriae give the bacterium the necessary anchorage to adhere to surfaces or form biofilms (Hahn 1997). The type 4 pili which are so extensively described in pathogenic strains are also thought to play a role in root adherence and biofilm formation (Dörr et al. 1998).

LPS of *Pseudomonas* and of most other Gram-negative bacteria consists of lipid A, core, and O-antigen. The later can consist of many repeating units. LPS of a pure culture is heterogeneous with respect to the number of repeating units in its O-antigen (Goldman and Leive 1980). Mutants devoid or defective in the O-antigenic side chain appear to be impaired in potato root colonization (de Weger et al. 1989). The ability to utilize amino acids which is an integral part of the root exudates confers a distinctive nutritional edge on a bacterial strain (Simons et al. 1997). Since root exudates are comprised mainly of monosaccharides and organic acids (Phillips and Streit 1995), mutants deficient in the utilization of individual crop-specific root exudates are impaired in root colonization, and hence one has to choose sets of beneficial microbe-plant pairs based on individual exudate composition and high bacterial growth rate on the major exudate carbon sources (Lugtenberg et al. 2001). Other rhizospheric competence traits that have been reported for *Pseudomonas* include putrescine utilization (Kuiper et al. 2001), protein secretion (Preston et al. 2001), enhanced outer membrane permeability (Lugtenberg et al. 2001), NADH functionality (Dekkers et al. 1998), and the ability to rapidly colonize root surfaces (O'Toole and Kolter 1998).

A new dimension of Pseudomonad utility in the agro-ecosystem is their ability to alleviate abiotic stress effects in plants. This has gained momentum over the years, and today *Pseudomonas* strains are known to confer tolerance to a variety of abiotic stresses (Grover et al. 2011). Of specific interest in this area is the ability of cold-adapted *Pseudomonas* strains to alleviate the frost injury/cold stress effects on plants (Xu et al. 1998; Mishra et al. 2011). This gains significance since the cropping cycle in several parts of the world is subject to transient cold periods, which are deleterious to plant beneficial microbial processes. Another emerging area is the alleviation of drought stress effects in plants by the action of inoculated osmotolerant *Pseudomonas* strains. A compilation of abiotic stress alleviation effects on various plants by *Pseudomonas* strains is presented in Table 10.3.

Table 10.3 Pseudomonads that alleviate abiotic stress effects in plants

Pseudomonas species	Effects/traits	Reference
P. fluorescens strain MSP-393	Tolerant to high osmolarity by the synthesis of novel proteins that nullify detrimental effects of drought	Paul et al. (2006)
P. fluorescens	Protects green gram plants from salt stress under in vitro conditions	Saravanakumar and Samiyappan (2007)
P. chlororaphis O6	Induced systemic drought and salt tolerance in asparagus plants	Cho et al. (2008)
P. mendocina	Tolerant to drought stress	Kohler et al. (2008)
Pseudomonas spp.	Promotes asparagus growth under water stress	Liddycoat et al. (2009)
Pseudomonas sp. strain AKM-P6	Increased heat tolerance of sorghum seedlings	Ali et al. (2009)
P. putida strain GAP-P45	Increased drought tolerance in sunflower	Sandhya et al. (2009)
P. mendocina	Promotes lettuce growth under elevated CO_2 and water stress conditions	Kohler et al. (2010)
P. putida Rs-198 strain	Protects and promotes cotton seedlings against salt stress	Yao et al. (2010)
Pseudomonas sp.	Confers drought tolerance to maize seedlings	Sandhya et al. (2010)
P. putida	Promotes wheat growth under high temperatures	Ali et al. (2011)
Pseudomonas spp.	Alleviates cold stress effects on wheat	Mishra et al. (2011)
Pseudomonas sp.	Drought tolerant and ACC deaminase producing	Ali et al. (2014)
Pseudomonas sp.	Thermotolerant trehalose synthase-producing strain	Ali et al. (2014)

(continued)

Table 10.3 (continued)

Pseudomonas species	Effects/traits	Reference
P. putida	Tolerates high temperature (45 °C) and salinity stress (1 M NaCl) through involvement of heat stress responsive molecular chaperones and membrane proteins during heat stress	Rangeshwaran et al. (2013)
P. chlororaphis	Confers salt stress tolerance in gladiolus in sodic soils	Damodaran et al. (2014)
Pseudomonas spp.	High freezing survival and low ice nucleation activity	Bisht et al. (2013)
Pseudomonas spp.	Tolerate salinity levels of 1.2 M. (7 % NaCl) and high temperature (50 °C)	Praveen Kumar et al. (2014)
Pseudomonas P59	Tolerates osmotic stress levels up to (−1.2 MPa)	Praveen Kumar et al. (2014)

Table 10.4 Pesticide degradation by Pseudomonads

Pseudomonas species	Functions	Reference
Pseudomonas sp.	Degrades atrazine by N-dealkylation and dehalogenation	Behki and Khan (1986)
P. putida	Utilizes methyl parathion as C-Source and produces the enzyme organophosphorus acid anhydrase	Lakshmi Rani and Lalitha kumari (1994)
Pseudomonas sp.	Enhanced biodegradation of γ-HCH upon 8 days of incubation	Nawab et al. (2003)
Pseudomonas sp.	Complete degradation of phorate was observed within 40 days	Bano and Musarrat (2003)
Pseudomonas spp.	Degradation of chlorpyrifos up to 71–77 % was recorded	Madhuri and Rangaswamy (2009)
P. fluorescens and *P. aeruginosa*	99 % degradation of chlorpyrifos was observed	Lakshmi and Prakash (2009)
Pseudomonas strain SFP 13	Recorded 70.2 % degradation of fenpropathrin	Sarkar et al. (2011)
P. aeruginosa (*S1*)	Degrades malathion	Tamer et al. (2013)
P. desmolyticum NCIM 2112	Degrades benzoyl benzoate	Rokade and Mali (2013)
Pseudomonas sp. ED1	Degrades endosulfan (250 ppm)	Siva and Rajam (2013)

Other Roles in the Agro-Ecosystem

The presence of organic pollutants such as pesticides and heavy metals above the permissible levels has adverse effects on plant growth and development and often renders the soils unfit for cultivation. Considering the variety of organic pollutants and heavy metals and the extent of cultivable land that are prone to such stresses, it's imperative to identify suitable and sustainable mechanisms of remediation. In such a scenario, *Pseudomonas* strains have the potential for remediation of contaminated environments due to their metabolic versatility (Aislabie and Lloyd-Jones 1995). The vast array of *Pseudomonas* enzyme systems confers upon them the ability to degrade organic pollutants such as pesticides (Foght et al. 2001), while the energy-dependent efflux pump mechanisms help them overcome stress imposed by heavy metals (Silver 1996). A non-exhaustive compilation of the instances of pesticide degradation and heavy metal tolerance by *Pseudomonas* strains is presented in Tables 10.4 and 10.5. Though the utility of Pseudomonads in this arena is well documented, their utility in the remediation of vast tracts of cultivable land is yet to be exploited.

Table 10.5 Heavy metal-tolerant *Pseudomonas* and their utility

Pseudomonas species	Functions/traits	Reference
P. fluorescens NCIMB 11764	Utilization of cyanide as a nitrogenous substrate	Kuntz et al. (1992)
P. fluorescens LB 300	Capable of reducing Cr (VI)	De Leo and Ehrlich (1994)
P. mendocina	Resistant to Cr (VI) up to 1,560 (mg/l)	Bhide et al. (1996)
Pseudomonas sp.	Resistance to mercury	Barbiere et al. (1996)
P. aeruginosa	Biosorption of uranium	Hu and Reeves (1997)
P. putida Spi3	Removal of mercury from electrolysis wastewater	Canstein et al. (1999)
Pseudomonas sp.	Biodegradation of cyanide compounds	Dhillon and Shivaraman (1999)
Pseudomonas sp.	Resistant to Cr (VI) up to 520 (mg/L)	McLean and Beveridge (2001)
P. aeruginosa strain BU2	Copper removal from aqueous solutions	Kazy et al. (2002)
P. fluorescens	Resistant to Cr (VI) up to 208 (mg/L)	Hussein et al. (2004)
P. putida	Resistant to Cr (VI) up to 156 (mg/L)	Hussein et al. (2004)
P. aeruginosa PU21	Biosorption of mercury	Chang and Hong (2004)
Pseudomonas sp.	Lanthanum biosorption	Kazy et al. (2006)
P. putida	Arsenic resistant strain	Rathinasabapathi et al. (2006)
P. brassicacearum	Stimulation of root growth and enhanced nutrient uptake under cadmium stress	Safronova et al.(2006)
P. aeruginosa strain BU2	Removal of uranium from aqueous solution	Kazy et al. (2008)
P. oleovorans	Resistant to Cr (VI) up to 780 (mg/l)	Mistry et al. (2009)
P. plecoglossicida	Resistant to Cr (VI) up to 600 (mg/l)	Poornima et al. (2010)
P. aeruginosa	Biosorption of metals Cr, Zn, and Cu	Nagashetti et al. (2013)
P. stutzeri ASP3	Resistant to arsenic (sodium arsenate up to 4,680.15 mg/l)	Shakya et al. (2012)

Conclusion

The genus *Pseudomonas* is unique and versatile in its metabolic and genome architecture and is therefore ubiquitous in distribution and varied in function. In the agro-ecosystem, though the plant growth promotion and related biological control properties of Pseudomonads are well studied, several other beneficial properties like the degradation of xenobiotic compounds and bioremediation of heavy metal-contaminated soils and environments remain as artifacts of academic interest only and have not seen much utility in terms of commercial exploitation. A recent interest on the abiotic stress alleviation properties of Pseudomonads is bound to generate interest in the emerging scenario of climate change and associated disturbances in the growing environments. As diversity of Pseudomonads continues to expand with time, it can be rightfully said that the attempts to unearth the real diversity of this genus have been clearly disproportionate, especially in the pristine environments such as the alpine and subalpine environments that are treasure troves of these unique bacteria. Since there is no dearth of basic information, the real need of the day is the unearthing of the undiscovered diversity and the viable commercial utilization of Pseudomonads for the welfare of mankind.

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Perspectives of Rhizobial Inoculation for Sustainable Crop Production

11

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Abstract

The need for sustainable agricultural practices is revitalizing the worldwide interest in biological nitrogen fixation (BNF) and rhizobia-legume symbiosis, particularly in organic production system. Rhizobia-legume symbiosis is a complex process involving a set of plant and bacterial genes leading to the formation and development of root nodules on legume roots where rhizobia fix nitrogen from the atmosphere. About 60 % of the Earth's available nitrogen is fixed via BNF. The success of the symbiotic process depends on the competitiveness, specificity, compatibility, and effectiveness of rhizobia with its host legume under variable soil and environmental conditions. In general, the interaction of rhizobia with nonlegumes has been neglected because of the lack of ability to respond to the rhizobial lipochitin Nod factors, which are the essential signal molecules that trigger legume nodulation. Work on rhizobial interaction with nonlegumes, over the years, has been done progressively; it has been demonstrated that rhizobia can associate with roots of nonlegumes also and can promote their growth by using one or more of the direct or indirect mechanisms of actions. The beneficial association/symbiosis of rhizobia can be negatively affected by many rhizosphere processes and other environmental stresses, resulting in important ecological, economic, and nutritional losses. The application of environment-friendly agricultural techniques, based on the use of plant growth-promoting rhizobacteria (PGPR) and/or plant growth regulators (PGRs), can increase the efficiency of the symbiotic/associative processes. The application of appropriate

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rhizobia together with PGPR/PGRs is considered an effective and environment-friendly approach which may profoundly increase the crop yield by different mechanisms of actions under variable conditions. Plant growth promotion of legumes/nonlegumes by rhizobia and their practical application as selected commercially available rhizobial inoculants are the major focus of this chapter.

Introduction

Most of the natural nitrogen inputs for life on Earth are fulfilled by biological reduction of molecular dinitrogen to ammonium. Such fixed nitrogen is acquired by the plants of the legume family through a symbiotic interaction with gram-negative soil bacteria called rhizobia. In the symbiotic interaction with rhizobia, legumes develop nodules which involve signal exchange between the host and the bacterium. Often ineffective nodulation in the legumes is experienced because of the competition of introduced rhizobial strains with the native rhizobial population and/or environmental stresses. In other words, the process of symbiotic nitrogen fixation is dependent on the efficiency and competitiveness of the rhizobial strain for nodulation against indigenous soil bacteria and environmental factors. Investigations have established that the use of compatible, efficient, and effective PGPR in combination with rhizobia could not only suppress/eliminate the effect of other rhizosphere strains and adverse environmental factors but also improve the performance of root-nodulating bacteria and hence nodulation, growth, and yield of the inoculated legumes. Also, the role of rhizobia as root colonizer of many nonlegumes is now well recognized. It has been established that rhizobia could express several characteristics like other PGPR. Rhizobia can increase the availability of plant nutrients and can produce various kinds of chemical compounds, namely, phytohormones, lipochitooligosaccharides (LCOs), lumichrome, siderophores, hydrogen cyanide (HCN), exopolysaccharides (EPS), and enzymes, which could improve plant growth and yield directly or indirectly. Rhizobial potential as biological control agents against many pathogens of nonlegumes has also been extensively documented. Rhizobia have also been reported as growth modifier in nonlegumes under stress conditions including salinity, temperature, drought, and heavy metal stress. Exploitation of rhizobia in combination with PGPR also constitutes an interesting alternative to improve their performance and association with crop plants under normal and stress conditions. Even more, it has been considered a promising avenue to optimize plant growth via managing interactions between PGPR, arbuscular mycorrhizal (AM) fungi, and the *Rhizobium*-legume symbiosis. Conclusively, this chapter presents a discussion on the *Rhizobium*-legume symbiosis and the mechanisms of action of rhizobia as PGPR to improve growth and yield of nonlegumes. The interactive effects of rhizobia with other PGPR and PGRs under normal and stress conditions to enhance crop production have been discussed. Finally, rhizobia-based commercially available inoculants have been highlighted.

Rhizobia-Legume Symbiosis: An Overview

Rhizobia-legume symbiosis is a highly coordinated effort between root-nodulating bacteria and the legume in soil for exchange of molecular dialogue (Parniske and Downie 2003). A leguminous plant initiates this symbiotic relationship with the release of certain compounds called flavonoids into the rhizosphere. In response to plant-released flavonoids, the special receptor (nodD) of the only specific rhizobia become activated. In turn, the nodD of rhizobia produces LCOs to sense and to recognize the chemicals released by the host legume roots (Russelle 2008). The LCOs in turn activate nodulins (a set of plant genes). The activated nodulins then

cause induction of various biochemical and morphological changes as a result of which cell division in the root cortex occurs (Oldroyd and Downie 2008) and a nodule meristem is thus formed within the root. Resultantly, the growth of root hair is redirected to curl and deform to entrap the attached rhizosphere rhizobia. The entrapped rhizobia start infections as a result of which plant produces a tube called infection thread. Through the infection thread, rhizobia enter into the root cortex and reach the nodule primordia. Then a plant-derived peribacteroid membrane quickly develops around the rhizobia after which the unit is called symbiosome. Within the symbiosome the bacteroids utilize the nitrogenase enzyme to catalyze the conversion of atmospheric N_2 to NH_3 (ammonia), a process called BNF. The bacteroids need large amounts of energy to support their nitrogen-fixing capacity which is provided by the host legume in the form of sugar produced through photosynthesis. It is estimated that the *Rhizobium*-legume symbiosis requires about 10 kg of carbohydrates (sugar) for each kg of N_2 fixed (Schulze 2004; Hubbell and Kidder 2009). Hence, in addition to having good health, the legume host must have enough water and other nutrients under proper sunlight. Likewise, fully effective nitrogen fixation requires rhizobial strains that are highly effective nitrogen fixers. Generally, nodules providing nitrogen to plants formed by effective rhizobia are usually large, often pink and red inside, whereas nodules formed by ineffective rhizobia tend to be white or light green inside, small, often quite numerous, and scattered throughout the root system. The ammonia formed as a result of *Rhizobium*-legume symbiosis is used to form amino acids which move out of the nodule via a compound provided by the plant, to other parts of the plant where it is used mainly to produce proteins.

Mechanisms of Plant Growth Promotion by Rhizobia

Rhizobium spp. could have an impact on plant growth and development in two different ways: directly or indirectly. The direct growth promotion of the plant by *Rhizobium* spp. generally entails providing the plant with compounds called phytohormones, regulating ethylene, solubilizing insoluble phosphates, and facilitating the uptake of nutrients from the environment. Rhizobia can benefit plant growth indirectly by decreasing or preventing some of the deleterious effects of phytopathogenic organisms through induced systemic resistance (ISR), parasitism, antibiosis, competition for nutrients, and production of metabolites suppressive to deleterious rhizobacteria. These mechanisms are discussed as under:

Direct Mechanisms of Growth Promotion

Rhizobia cause promotion in plant growth directly by regulating plant ethylene levels; producing plant hormones; providing useful compounds like lumichrome, riboflavin, and LCOs; sequestering iron by releasing siderophores; and solubilizing insoluble phosphorus via releasing organic acids and enzymes (Mehboob et al. 2009).

Ethylene, a plant hormone, generally shows enhancement in root initiation and growth at its low level, but its higher levels can lead to suppression in root elongation (Shaharoona et al. 2006a). Usually increased ethylene level damages/retards growth of plants facing a variety of environmental stresses like heavy metals, pathogens, drought, and salinity. The enzyme ACC deaminase has been known to hydrolyze ACC (the immediate biosynthetic precursor of ethylene in plants) into ammonia and α-ketobutyrate which can be used by the bacterium as a source of nitrogen and carbon. Several rhizobial strains investigated to produce ACC deaminase enzyme including *Rhizobium japonicum*, *Rhizobium leguminosarum*, *Rhizobium hedysari*, *Rhizobium gallicum*, *Bradyrhizobium japonicum*, *Bradyrhizobium elkanii*, *Mesorhizobium loti*, and *Sinorhizobium meliloti* could stimulate the growth of plants by regulating their ethylene levels upon inoculation (Ma et al. 2004; Uchiumi et al. 2004; Nukui et al. 2006; Hafeez et al. 2008; Duan et al. 2009).

Plant hormones are not nutrients but are chemicals that in small amounts promote and influence the growth and development. In general, it is accepted that there are five major classes of

plant hormones, namely, abscisic acid, auxins, cytokinins, ethylene, and gibberellins. Other plant hormones and growth regulators such as brassinosteroids, salicylic acid, jasmonates, plant peptide hormones, polyamines, nitric oxide, strigolactones, and karrikins are not easily grouped into these classes; they exist naturally or are synthesized by other organisms that interrupt the physiological processes within plants. Each hormone has positive and inhibitory functions. It is now well established that there are two sources of phytohormones naturally available for plants: endogenous production by the plant tissues and exogenous production by associated microorganisms. Auxins, abscisic acid, cytokinins, gibberellins, ethylene, and nitric oxide are the most studied plant hormones released by *Rhizobium* spp. during their interaction with plants (Khalid et al. 2006; Perrine-Walker et al. 2007). *Azorhizobium caulinodans*, *B. japonicum*, *M. loti*, *R. leguminosarum*, and *S. meliloti* are the examples of studied strains promoting plant growth via producing various phytohormones (Naidu et al. 2004; Dazzo et al. 2005; Mishra et al. 2006; Boiero et al. 2007; Chandra et al. 2007; Pandey and Maheshwari 2007; Afzal and Bano 2008; Vargas et al. 2009; Chi et al. 2010).

Lumichrome is a signaling compound that can affect plant growth in both natural and agricultural ecosystems (Beveridge et al. 2003). Rhizosphere accumulation of lumichrome has been reported to act as developmental signal (Kanu and Dakora 2009). Lumichrome, a compound that is produced by rhizobia, acts as an enhancer of plant growth prior to the onset of nitrogen fixation (Cooper 2007). Lumichrome is a flavin identified from *Rhizobium* spp. which enhanced nodule number when applied to lotus (*Lotus japonicus*) (Gouws et al. 2012). The stimulation of seedling development in cereal, by lumichrome, has also been recognized (Matiru and Dakora 2005). Also, it is known that lumichrome could orchestrate plant development. It has been identified from culture filtrate of *S. meliloti* cells (Dakora 2003). Moreover, rhizobial inoculation of plants has been suggested to alleviate the effect of water stress through the production of lumichrome (Phillips et al. 1999).

Riboflavin is a vitamin that enzymatically or photochemically changes into lumichrome which in turn stimulates plant growth via motivating root respiration (Phillips et al. 1999; Dakora et al. 2002). It is a necessary component of the flavin coenzymes FMN and FAD. It has been reported that riboflavin could also act as a signal of development upon accumulation in the rhizosphere (Kanu and Dakora 2009). *Rhizobium*, *S. meliloti*, and *R. leguminosarum* bv. *viciae* have been recognized to produce riboflavin as growth promoter of plants (Yang et al. 2002; Dakora et al. 2002).

LCOs commonly known as Nod factors are bacterium-to-plant signal molecules produced by rhizobia during rhizobia-legume symbiosis (Spaink 1992). These have been shown to induce rapid and transient alkalinization of plant cells and to restore or resume cell division and embryo development in various plants (De Jong et al. 1993; Staehelin et al. 1994; Baier et al. 1999; Dyachok et al. 2000). Seed germination, root mass, and length enhancement could also result via rhizosphere application of LCOs at low concentration (10^{-7} to 10^{-9} M). The production of photosynthate and grain yield could also be increased via leaf spray of LCOs in micromolar concentration, i.e., 10^{-6} to 10^{-10} (Miransari and Smith 2009). *Rhizobium* spp. are also known for the induction in expression of flavonoid genes, cell division, and embryogenesis via releasing LCOs (Smith et al. 2002).

Iron, a plant nutrient needed for chlorophyll synthesis, is often present in a highly insoluble form in soil. Deficiency of iron causes initial yellow or white areas between the veins of young leaves leading to spots of dead leaf tissues. Siderophore, a low molecular weight compound, is produced to sequester Fe^{3+} as well as to mobilize it in soil. Sequestration and transportation of iron into plant cell via siderophores by rhizobia is assumed to serve as a source of plant-available iron under iron-deficient environment. Several *Rhizobium* spp. have been known to bind insoluble Fe^{3+} and reduce it to soluble and plant-preferred form, i.e., Fe^{2+} (Antoun et al. 1998; Carson et al. 2000; Arora et al. 2001).

Phosphorus (P) is a major and key nutrient limiting plant growth (Podile and Kishore 2006).

A large portion of applied and soil phosphorus becomes unavailable to plants because of its immobilization in either organic or inorganic form due to low or high pH of soil. At low pH, P is fixed in soil by free oxides and hydroxides of Al and Fe, while at high pH it is fixed by Ca. Generally, rhizosphere bacteria convert the fixed/insoluble phosphates into soluble form through the process of acidification, chelation, and exchange reactions and production of gluconic acid (Rodriguez et al. 2004; Chung et al. 2005; Hameeda et al. 2008). *Rhizobium* spp. have the ability to mobilize the bound phosphorus either by releasing enzymes, phosphatases, or organic acid, namely, citric acid, glutamic acid, carbonic acid, and gluconic acid (Abd-Alla 1994; Alikhani et al. 2006; Jayasinghearachchi and Seneviratne 2006; Afzal and Bano 2008). *B. japonicum*, *R. leguminosarum*, *S. meliloti*, and *Mesorhizobium mediterraneum* are rhizobia that have been investigated to solubilize or mineralize phosphate in the rhizosphere of plants (Egamberdieva et al. 2004; Hara and de Oliveira 2004; Puente et al. 2004; Rodriguez et al. 2006; Fernandez et al. 2007; Afzal and Bano 2008).

Eighteen elements have been declared as essential for plants, each of which has at least one specially defined role in plant growth. *Rhizobium* spp. could contribute significantly in maintaining adequate nutrient uptake from soils by reducing the accumulation of nutrients in agricultural soils. Several studies can be cited about the relations with *Rhizobium* spp. and enhancement of nutrient uptake. For example, Khokhar and Qureshi (1998), Biswas et al. (2000), and Yanni et al. (2001) have reported significantly increased uptake of N, P, K, Ca, Mg, Zn, Na, Mo, and Fe by rice due to inoculation with *Rhizobium* spp. Similarly, Hafeez et al. (2004) demonstrated increased uptake of K and Ca by cotton due to *R. leguminosarum* bv. *trifolii* and enhanced uptake of N, K, Na, Zn, Fe, and Cu by wheat due to *Rhizobium* inoculation (Amara and Dahdoh 1997).

Conclusively, growth and yield of leguminous crops could be triggered by rhizobial inoculation directly via producing various metabolites/substances such as plant hormones, ACC deaminase enzymes, LCOs, siderophores, lumichrome, riboflavin, etc., and/or by fixing/solubilizing/increasing the uptake of mineral nutrients.

Indirect Mechanisms of Growth Promotion

Rhizobia also exhibit several indirect mechanisms of plant growth promotion which involve antibiosis, parasitism, competition for nutrients, and induction of systemic resistance.

Antibiosis is one of the effective mechanisms of rhizobia to prevent the proliferation of phytopathogens via synthesizing antimicrobial compounds known as antibiotics. Many rhizobial species have been reported to secrete antibiotics and cell wall-degrading enzymes that can inhibit/kill the phytopathogen (Siddiqui and Mahmood 2001; Shaukat and Siddqui 2003; Bardin et al. 2004; Chandra et al. 2007; Mabrouk and Belhadj 2010).

Parasitism is a type of symbiosis in which one organism (called parasite) benefits at the expense of another organism usually of different species (called host). The association may also lead to the injury or death of the host. The mechanism of parasitism has been used successfully by *Rhizobium* spp., namely, *R. leguminosarum*, *S. meliloti*, and *B. japonicum*, against fungal pathogens belonging to genera *Macrophomina*, *Rhizoctonia*, and *Fusarium* (Siddiqui et al. 2000; Ozkoc and Deliveli 2001).

A contest in which a winner is selected from among two or more parties is called competition. It is believed that a phytopathogen could be displaced through competition mechanism between rhizobia and pathogen. The best understood example of competition is that for iron. Siderophores are secreted by the rhizobial complex with available iron in the rhizosphere to make it less available to competing phytopathogens. More clearly, in iron competition, the biocontrol agent produces siderophores that sequester iron in the rhizosphere and make it less available to certain deleterious rhizosphere microorganisms. The latter cannot obtain sufficient iron for growth and thus are outcompeted. In other words, plant growth and development could be triggered by biocontrol agents indirectly through starvation by

producing siderophore. *Rhizobium* spp. are able to produce siderophores and can inhibit a widely occurring plant pathogen, *Macrophomina phaseolina* (Arora et al. 2001). Various *Rhizobium* species, namely, *R. leguminosarum* bv. *viciae*, *R. leguminosarum* bv. *trifolii*, *R. leguminosarum* bv. *phaseoli*, *R. meliloti*, and *R. tropici*, are able to produce siderophores in iron-deficient environment to chelate it (Carson et al. 2000; Arora et al. 2001).

To make the host plant more resistant to pathogens via inducing a change in its susceptibility is termed as ISR. Rhizobia are able to control pathogens by induction of plant defense mechanisms (Abdelaziz et al. 1996). Several studies have demonstrated the improvements in defense mechanisms of plant via release of biostimulatory agents by rhizobia (Peng et al. 2002; Singh et al. 2006). *Rhizobium etli*, *R. leguminosarum* bv. *phaseoli*, and *R. leguminosarum* bv. *trifolii* have been reported to provide bioprotection during pathogenic stresses via induction of enhanced systemic resistance in potato and rice plants (Reitz et al. 2000; Mishra et al. 2006).

In conclusion, rhizobia can indirectly promote the growth of legumes by inhibiting the phytopathogens via producing antibiotics and fungal cell wall-degrading enzymes and HCN or through starvation via siderophore production.

Synergistic Interaction of Rhizobia with Beneficial Microorganisms: Improving Biomass and Nodulation

A synergistic interaction is an interaction in which two or more elements/parties work together in a system to produce an effect greater than the sum of their individual effects. Figure 11.1 illustrates various strategies to improve rhizobia-legume

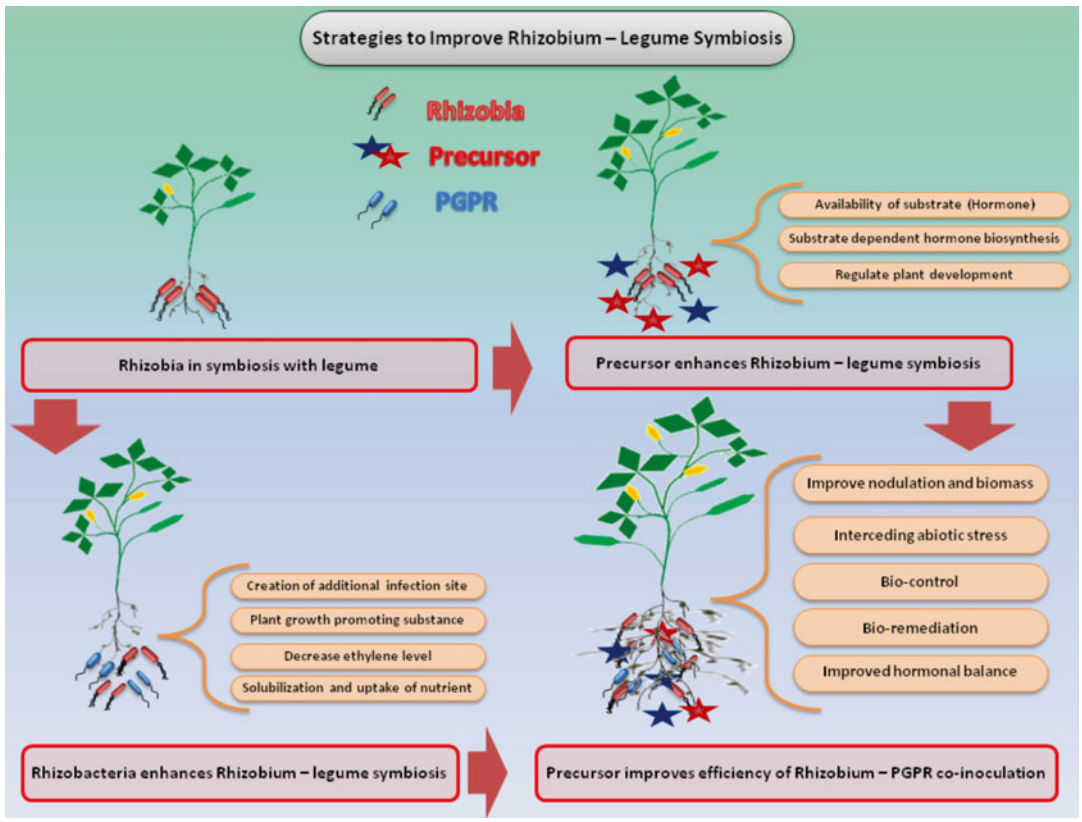

Fig. 11.1 Schematic view of different strategies to improve *Rhizobium*-legume symbiosis

symbiosis. As microbial inoculant formulations containing single strain have often shown inconsistent results, emphasis has been given on co-inoculations in which a co-inoculant interacts synergistically to improve the performance of other beneficial microorganisms (Bashan and de-Bashan 2005). Table 11.1 depicts examples of the synergistic impact of co-inoculation of rhizobia with other PGPR. Synergistic effects of rhizobia when used in combination with beneficial microorganisms have been focused in detail below.

Plant Growth-Promoting Rhizobacteria

Studies have recognized the improvement in plant biomass and nodulation by co-inoculation of rhizobia with PGPR. However, the ability of co-inoculant PGPR depends on the inoculant strain's inherent potential and effectiveness/specificity to certain plant species, cultivar, and genotype, composition of root exudates of host plant, cell density of applied inocula/optimal inoculation dose, temperature variation, and interaction of applied inocula with rhizosphere microflora predominant in the particular crop (Medina et al. 2003; El-Sawy et al. 2006; Remans et al. 2008; Mishra et al. 2009; Fox et al. 2011). Some PGPR from a range of genera improve plant growth and yield when used in combination with rhizobia. Some PGPR from various genera, which improved plant biomass and nodulation when used in combination with rhizobia, are the major focus in the following review:

Azospirillum, a free-living N_2 fixer and a PGPR, has been recognized for its ability to improve nodulation and biomass of legumes as a co-inoculant of rhizobia (Roseline et al. 2008). Improved rhizobial infection, nodule formation,

Table 11.1 Rhizobial inoculation in combination with rhizobacteria or arbuscular mycorrhizal fungi to improve nodulation and biomass of legumes

Rhizobia	Co-inoculants	Host plant	Proposed mechanism(s)/plant response	References
Rhizobium spp. strain Mg6	PGPR strains A1 and A2	*Phaseolus vulgaris*	ACC deaminase activity	Aamir et al. (2013)
Rhizobium leguminosarum bv. *viciae*	Arbuscular mycorrhizal fungi	*Vicia faba*	Mobilization of P, Fe, K, and other minerals	Abd-Alla et al. (2014)
Rhizobium sp.	PGPR, P-enriched compost	*Cicer arietinum*	Improved growth and nodulation through ACC deaminase activity	Shahzad et al. (2014)
B. japonicum strains MN-S and TAL-102	AM fungi, *Glomus intraradices*	*V. radiata*	Significant improvement in plant biomass and N contents	Yasmeen et al. (2012)
Rhizobium sp. strain PK20	*Pseudomonas* sp. M9	–	ACC deaminase activity of *Pseudomonas* sp. M9	Ahmad et al. (2011, 2013)
R. leguminosarum-PR1	*Pseudomonas* sp. strain NARs1	*Lens culinaris*	Increased growth and nutrient uptake	Mishra et al. (2011)
R. leguminosarum	*Pseudomonas* spp.	*V. faba*	P solubilization, phytohormone and siderophore production	Saidi et al. (2013)
Mesorhizobium sp. BHURC03	*Pseudomonas aeruginosa* BHUPSB02	*C. arietinum*	Enhanced P and Fe uptake, nodulation, and production of IAA	Verma et al. (2013)
R. leguminosarum	PGPR, enriched compost	*L. culinaris*	ACC deaminase activity of PGPR and symbiotic competence of rhizobia	Iqbal et al. (2012)
Rhizobium sp.	*Pseudomonas* sp. LG or *Bacillus* sp. Bx	*P. vulgaris*	P solubilization, IAA, ammonia, and siderophore production	Stajković et al. (2011)

and N_2 fixation activity as a result of the combined inoculation with *Azospirillum* and *Rhizobium* via the enhanced production of plant hormones and siderophores and enhancement of total mineral uptake have been claimed as a mechanism for growth and biomass enhancement of various legumes (Bashan 1998; Wani et al. 2007a; Dardanelli et al. 2008; Cassan et al. 2009). Numerous studies have demonstrated the enhancement of nodulation and growth of a wide array of legumes because of the positive interaction between rhizobia and bacteria of the genus *Azospirillum*, namely, in white clover (Tchebotar et al. 1998), faba bean and chickpea (Rodelas et al. 1999; Wani et al. 2007b), pigeon pea (Deanand et al. 2002), and soybean (Cassan et al. 2009). Species of *Azospirillum*, viz., *A. lipoferum*, *A. brasilense*, *A. amazonense*, *A. halopraeferens*, and *A. irakense*, have been identified so far as co-inoculant of rhizobia (Tarrand et al. 1978; Magalhaes et al. 1983; Reinhold et al. 1987; Khammas et al. 1989).

Bacillus, one of the most familiar soil bacterial groups, has been found to be an effective PGPR. The potential of *Bacillus* together with rhizobia as a promoter of nodulation and biomass of various legumes via improving rhizobial colonization, nodule occupancy, choice of changing nodulation competition between strains, number of infection sites, production of phytohormones and siderophores, biocontrol of diseases, and efficient uptake of nutrients has been investigated (Vessey and Buss 2002; Yuming et al. 2003; Elkoca et al. 2008; Rajendran et al. 2008; Mishra et al. 2009; Medeot et al. 2010; Tsigie et al. 2012; Atieno et al. 2012). Enhanced root and shoot number, length, surface area, nodulation, nodule number, dry weight, and plant height and branches per plant are the reported stimulatory effects of co-inoculation of some of the *Bacillus* strains with rhizobia on various legumes (Guinazu et al. 2010; Stajkovic et al. 2011; Singh et al. 2011).

Another most studied plant PGPR is *Pseudomonas* which belongs to Gram-negative, rod-shaped, nonspore-forming bacteria. Species of *Pseudomonas* including *P. fluorescens* 2137, BHUPSB06, and P-93; *Pseudomonas* sp. strain PGERs 17, FM7d, MRS 13, MRS 16, CDB 35, BWB 21, LG, CPS63, and MPS; *P. striata*; *P. putida*; *P. trivialis* 3Re27; *P. maltophilia*; and *P. jessenii* PS06 have been found to have a role in improving nodulation and biomass of various legumes when used in combination with rhizobia. Root/shoot length, surface area, nitrogenase activity, N_2 fixation, total N content, and plant dry weight are the features of plants which were improved by *Pseudomonas* when used in combination with rhizobia (Deanand et al. 2002; Zaidi et al. 2003; Garcia et al. 2004; Tilak et al. 2006; Malik and Sindhu 2008; Stajkovic et al. 2011; Mishra et al. 2012; Verma et al. 2012).

Azotobacter, free-living, aerobic, N_2-fixing bacteria, is another PGPR which has shown increases in nodulation, plant biomass, and yield of various legumes, namely, lentil, urdbean, soybean, clover, chickpea, and peanut, as a result of its dual inoculation with *Rhizobium* (Chandra and Pareek 2002; Qureshi et al. 2009; Dashadi et al. 2011; Akhtar et al. 2012).

Also, a PGPR, namely, *Serratia*, has proven its ability to invigorate nodulation and biomass of legumes in combination with rhizobia. Increase in root proliferation, infection sites, number and mass of nodule in various legumes are the demonstrated activities of *Serratia* when used in combination with rhizobial strains (Bai et al. 2002; Radwan et al. 2005; Badawi et al. 2011; Zahir et al. 2011).

Increased nodule occupancy and grain yield of various legumes has been reported due to another PGPR, *Enterobacter*, when used in combination with *Rhizobium* (Gupta et al. 1998; Mirza et al. 2007; Mishra et al. 2009).

Arbuscular Mycorrhizal (AM) Fungi

Another promising avenue to optimize plant growth is through managing interactions between plant growth-promoting bacteria (PGPB), AM fungi, and the *Rhizobium*-legume symbiosis. Perhaps fortuitously, the mycorrhizal fungi that are known to form associations with more than 80 % of plant species often enhance nutrient and water uptake. PGPB are able to increase AM

fungal development by affecting root colonization as well as by enhancing plant N and P uptake (Richardson et al. 2009). Production of EPS by PGPB significantly enhances the attachment of bacteria to mycorrhizal roots and AM fungal structures that influence the movement of bacteria in the rhizosphere (Bianciotto et al. 2001). Soil microbes are able to enhance the amounts of root exudates resulting in the activation of AM hyphae and hence higher rate of root colonization (Barea et al. 2005). Meyer and Linderman (1986) reported higher nodulation and shoot dry weight in clovers as a result of dual inoculation with AM fungi and PGPB as compared to inoculation with mycorrhizae or PGPB alone, whereas Azcon (1993) reported maximum growth and nutrient uptake as a result of the addition of PGPB cell-free culture to the mycorrhizal and nodulated legume *Hedysarum coronarium*. Silveira et al. (1995) revealed increased nodulation, root growth, and nitrogen and phosphorus uptake by beans as a result of mixed inoculation of fluorescent *Pseudomonas* and *Glomus etunicatum*. Vivas et al. (2003) reported enhanced shoot biomass from 18 % (at 13.6 mg Cd kg^{-1} soil) to 35 % (at 85.1 mg Cd kg^{-1} soil) and N, P, Zn, and Ni contents and transfer of reduced Cd from soil to plants by up to 37.5 % as a result of combined use of indigenous AM fungi plus indigenous PGPB in an experiment with clover grown in Cd-contaminated soil.

However, further research is needed for detailed illustration of interactions between the host plant, AM fungi ,and nodular bacteria by using different molecular techniques so as to enhance the ecosystem productivity.

Rhizobium spp. Containing ACC Deaminase for Improving Nodulation and Biomass

Ethylene production in the infected roots enhances as a result of *Rhizobium* invasion into legume roots during the nodulation process because of which the inhibition of nodulation in various legumes (viz., alfalfa, mung bean, *Pisum sativum*, and *Medicago sativa*) has been reported (Lee and LaRue 1992; Caba et al. 1998). Though enhancement of nodulation in various legumes by lowering ethylene levels via chemicals like aminooxyacetic acid (AOA), L-α-(aminoethoxyvinyl)-glycine (AVG), and rhizobitoxine has been established, it has also been documented (Nukui et al. 2000; Tamimi and Timko 2003) that there are rhizobial strains having an enzyme ACC deaminase which could reduce this stress by decreasing the level of ethylene in the host plant (Ma et al. 2003b). Hence, isolation and introduction of efficient rhizobial cultures with ACC deaminase have been stressed by Elsheikh and Ibrahim (1999) in order to improve the nodulation status, seed quality, and legume productivity.

Strains of *R. leguminosarum* having ACC deaminase action were more effective for pea nodulation (Ma et al. 2003b). Likewise, *S. meliloti* containing ACC deaminase gene (*acdS*) derived from *R. leguminosarum* also indicated increased capability to nodulate alfalfa (Ma et al. 2004). Uchiumi and colleagues (2004) showed that the inactivation of the gene *acdS* in *M. loti* resulted in a decreased number of nodules on *L. japonicus*, in comparison with the number of nodules formed by the wild-type strain.

Brígido et al. (2013) evaluated the symbiotic performance of two *Mesorhizobium ciceri* strains, transformed with an exogenous 1-aminocyclopropane-1-carboxylate deaminase gene (*acdS*), in chickpea plants under salinity stress. The EE-7 (salt-sensitive) and G-55 (salt-tolerant) *M. ciceri* strains were transformed with an *acdS* gene present on plasmid *pRKACC*. They recorded a significantly higher shoot dry weight in plants inoculated with the *acdS*-transformed salt-sensitive strain compared with the plants inoculated with the native strain in the presence of salt. The negative effects of salt stress were reduced when using *acdS*-transformed strains in comparison with the wild-type strains. They further demonstrated that in expressing the exogenous *acdS* gene, the salt-sensitive strain was able to induce nodules to the same extent as the salt-tolerant strain. Similarly, Nascimento et al. (2012) described that rhizobial strains expressing the enzyme ACC deaminase displayed an augmented symbiotic performance as a consequence

Table 11.2 Rhizobial spp. having ACC deaminase for improving legume crops

Rhizobium	Host legume	References
Rhizobia, PGPR	*V. radiata*	Aamir et al. (2013)
R. phaseoli	*V. radiata*	Ahmad et al. (2011)
Rhizobium radiobacter, *Mesorhizobium* sp.	*Salicornia brachiata*	Jha et al. (2012)
R. leguminosarum Biovar *viciae*	*P. sativum*	Ma et al. (2003b)
S. meliloti	*M. sativa*	Ma et al. (2004)
Bradyrhizobium isolate GSA11 or rhizobial isolate GSA6	*Cyamopsis tetragonoloba*	Khandelwal and Sindhu (2013)
Bradyrhizobium sp.	*V. radiata*	Shaharoona et al. (2006b)
Methylobacterium fujisawaense	*Brassica campestris*	Madhaiyan et al. (2007)

of lowering the plant ethylene levels that inhibits the nodulation process in chickpea.

Enhanced nodulation in various legumes, namely, chickpea, alfalfa, peas, *Medicago truncatula*, and *L. japonicus*, by various rhizobial strains (viz., *R. leguminosarum* biovar *viciae*, *S. meliloti*) as a consequence of reduction in ethylene synthesis has been documented by various researchers (Nukui et al. 2000; Mann et al. 2002; Ma et al. 2003a, 2004; Prayitno and Mathesius 2010). Likewise, Khandelwal and Sindhu (2013) reported increased number of nodules in cluster bean by inoculation at 60 days of growth under sterilized chillum jar conditions with ACC^+ *Bradyrhizobium* isolate GSA11 compared with *Rhizobium* isolate GSA6 having no ACC deaminase activity and suggested that the use of ACC deaminase-containing rhizobial bacteria could result in improved nodulation and could have growth-promoting effects leading to improved legume productivity. Therefore, the exploitation of ACC utilizing rhizobacterial isolates as bioinoculants for improving nodulation and plant growth of legumes has been emphasized. Table 11.2 enlists some of the rhizobial species reported for ACC deaminase activity for improving crops.

Performance of *Rhizobium* spp. Under Stress Environment

Ever since the colonization of plants on land, they have been facing extreme conditions. For the sake of survival, plants have evolved certain mechanisms in response to posed environmental stresses and made them competent inhabitants. Although many plants lack the ability to adapt to stress conditions, several plant species require microbial associates to ensure their survival and tolerance against stress. The plant's responses to environmental stresses vary due to microbial association. Therefore, it is inevitable to study the microbial interaction with plants, particularly PGPB which live with these plants synergistically and help in survival under constrained environments. Rhizobia can improve crop production under normal and constrained conditions. These have also been reported as plant growth promoters in nonlegumes where they perform different plant growth-promoting activities to enhance plant growth under normal and stress conditions including drought, salinity, temperature, heavy metal, and biotic stresses. These rhizobial associations may modify the physiology and biochemistry of crop plants which could lead to sustainable agriculture. The performance of rhizobia and their association with crop plants under drought, salinity, temperature, heavy metal, and biotic stresses has been reviewed and described in this section as well as in Table 11.3.

Drought Stress

Rhizobia have been reported for their ability to survive under water stress up to −1.5 and −3.5 MPa by Elboutahiri et al. (2010) and Abolhasani et al. (2010), respectively. Rhizobia

Table 11.3 Rhizobial inoculation for ameliorating impact of different stresses in crop plants

Rhizobium spp.	Host plant	Proposed mechanism(s)/plant response	Reference
Drought stress			
Rhizobium spp.	*Zea mays*, *Triticum aestivum*	Induced drought tolerance through catalase, exopolysaccharide, and IAA production	Hussain et al. (2014a, b)
Rhizobium spp., *Glomus mosseae*, *Glomus intraradices*	*P. vulgaris*, *Z. mays*	Improved growth and yield and alleviated moderate drought stress in combination	Franzini et al. (2013)
Rhizobium gallicum 8a3	*P. vulgaris*	Regulated water relations in plant	Sassi-Aydi et al. (2012)
Ensifer meliloti 4H41	*C. aeritinum*	Induced stress-adapted metabolic activities and constructive adaptation for specific cortex structure	Mhadhbi et al. (2011)
B. japonicum (Thal-8, Tal 620, Dulawala)	*C. aeritinum*	Synthesized gibberellic acid (GA), indoleacetic acid (IAA), and abscisic acid (ABA)	Bano et al. (2010)
Mesorhizobium ciceri	*C. aeritinum*	Production of ascorbate peroxidase (APX) and peroxidase (POX)	Esfahani et al. (2010)
Rhizobium sp.	*C. aeritinum*	Induced production of antioxidants	Mhadhbi et al. (2008)
R. etli	*P. vulgaris*	Production of trehalose-6-phosphate	Suárez et al. (2008)
Rhizobium sp.		Capability to survive under drought	Mahmood and Athar (2008)
Salinity stress			
Mesorhizobium tianshanense, *G. intraradices*	*Lotus tenuis*	Modulation of proline and polyamine	Echeverria et al. (2013)
Rhizobium galegae HAMBI 1141, *Pseudomonas extremorientalis* TSAU20, *Pseudomonas trivialis* 3Re27	*Galega officinalis*	Improved root tip colonization	Egamberdieva et al. (2013)
S. meliloti	*M. sativa*	Proline accumulation in nodules	López-Gómez et al. (2014)
Rhizobium strains RhOF4 and RhOF6	*V. faba*	Regulated enzymes of the ascorbate-glutathione cycle and the reduced glutathione (GSH)	Oufdou et al. (2014)
R. tropici CIAT899, *R. gallicum* RhM11	*P. vulgaris*	Acid phosphatase and trehalose-6-phosphate phosphatase activity	Faghire et al. (2013)
Rhizobia	*Arachis hypogaea*	Improved shoot growth under salinity	El-Akhal et al. (2013)
Rhizobium, arbuscular mycorrhizal fungi	*Acacia saligna*	Increased uptake and solubilization of nutrients	Soliman et al. (2012)
R. leguminosarum bv. *ciceri*		Production of mucus and organic acid	Öğütçü et al. (2010)

(continued)

Table 11.3 (continued)

Rhizobium spp.	Host plant	Proposed mechanism(s)/plant response	Reference
Temperature stress			
Mesorhizobium sp.	Not described	Increased transcriptional induction of chaperone genes	Alexandre and Oliveira (2011)
Bradyrhizobia strains	*Glycine max*	Efficient nitrogen fixation at high temperatures	Rahmani et al. (2009)
Rhizobium sp.	*Prosopis juliflora*	Improved symbiosis and nitrogen fixation	Kulkarni and Nautiyal (2000)
Sinorhizobium sp.	*Acacia senegal*	Competent colonization and nodulation ability	Raësaënen and Lindstroëm (1999)
Rhizobium sp.	*P. vulgaris*	Increased uptake of methionine and protein synthesis	Michiels et al. (1994)
Heavy metal stress			
B. japonicum E109	*Glycine max*	Reduced symbiosis due to As toxicity	Talano et al. (2013)
Rhizobium, *Sinorhizobium* spp.	*Lathyrus sativus*, *Lens culinaris*, *Medicago marina*, *M. truncatula*, *M. minima*	Phytoremediation of Cd-contaminated soil	Guefrachi et al. (2013)
Cupriavidus necator	*Leucaena leucocephala*, *Mimosa pudica*, *Mimosa caesalpiniaefolia*	Bioremediation of Zn-, Cu-, Pb-, and Cd-contaminated soils	Ferreira et al. (2013)
S. meliloti	*M. sativa*	Promotes the bioaccumulation of Zn in nodulated roots	Zribi et al. (2013)
Rhizobium spp.	*L. culinaris*	Decreased uptake of Ni in contaminated soil and improved plant growth	Wani and Khan (2013)
Mesorhizobium spp.	*Anthyllis vulneraria*	Decreased Zn concentration in both roots and shoots	Soussou et al. (2013)
Bradyrhizobium sp.	*V. radiata*	Survive under Zn stress and developed efficient symbiosis	Pongsilp and Boonkerd (2011)
Azorhizobium caulinodans	Not described	Avoidance of excessive Cd through absorption with lipopolysaccharides and cadmium extrusion	Zhengwei et al. (2005)
Biotic stress			
Rhizobia	*C. arietinum*	Suppression of *Rhizoctonia solani*	Hemissi et al. (2013)
R. leguminosarum strain RhOF4	*V. faba*	Reduction in the impact of cyanotoxin biohazard	Lahrouni et al. (2013)
Mesorhizobium loti	*Brassica campestris*	Suppression of white rot disease/*Sclerotinia sclerotiorum*	Chandra et al. (2007)
R. leguminosarum bv. *phaseoli* strain RRE6, R. *leguminosarum* bv. *trifolii* strain ANU843	*Oryza sativa*	Suppression of *Rhizoctonia solani*	Mishra et al. (2006)
R. etli strain *G12*	Potato	Suppression of cyst nematode (*Globodera pallida*)	Reitz et al. (2000)
R. meliloti	Sunflower, okra	Suppressed *Macrophomina phaseolina*, *Rhizoctonia solani*, *Fusarium solani*	Ehteshamul-Haque and Ghaffar (1993), Arora et al. (2001)
R. leguminosarum bv. *viciae*	Sugar beet	Suppressed pythium/ damping-off disease	Bardin et al. (2004)

probably produce antioxidants, osmolytes, and stress proteins to survive under harsh environments. The ability of these beneficial bacteria to tolerate severe water-deficit conditions can be utilized to ameliorate drought impact on plants (Goyal et al. 1986). They can induce stress tolerance in plants by producing physical and chemical changes (Yang et al. 2009). Different mechanisms for stress amelioration and plant growth promotion by rhizobia have been observed including production of chaperones and sugars (Berjak 2006), synthesis of stress enzyme ACC deaminase (Zahir et al. 2009), EPS production (Alami et al. 2000), production of low molecular weight organic compounds like trehalose (Zahran 1999), phosphate solubilization (Zaidi et al. 2009), improved nutrient availability (Hussain et al. 2009), production of siderophores (Arora et al. 2001), phytohormone production (Khalid et al. 2006), and enhanced root respiration (Volpin and Phillips 1998) by influencing plant physiology.

Rhizobia can relieve the effect of water-deficit stress by altering root morphology, carbon assimilation rate, transpiration rate, and leaf stomatal conductance in inoculated non-nodulating crops (Chi et al. 2005). A significant improvement in root/shoot length and nutrient and water use efficiency of sunflower seedlings was recorded due to *Rhizobium* species (strain YAS34) inoculation under drought by Alami et al. (2000). They suggested that the improvement was due to the EPS-producing ability of the strain YAS34. Sandhya et al. (2009) recorded substantial capability of rhizobia for improving water-holding capacity of the rhizosphere by developing microaggregates and biofilm around the plant roots to improve nutrient uptake and water flow. In the same way, Kaci et al. (2005) isolated the rhizobial strain KYGT207 from the drying soil and measured improvement in wheat growth under water-deficit conditions due to better rhizosphere soil aggregation. Similarly, Belimov et al. (2009) recorded improvement in drought-abiding ability of potato by the inoculation of PGPB containing ACC deaminase in a pot trial. Zahir et al. (2008) measured improved water use efficiency in plants inoculated with ACC deaminase producing PGPB due to increase in root length. Microbial chaperones have also been reported to improve plant growth under desiccated conditions by Castiglioni et al. (2008). They inoculated chaperones producing bacteria to maize and observed water-deficit stress alleviation in maize. Forchetti et al. (2010) isolated PGPB from sunflower and measured their ability to produce salicylic acid under drought. They also observed phosphate solubilization and oxidase, catalase, and jasmonic acid production ability of these PGPB. They concluded that bacteria having capability to produce antioxidants and osmoprotectants can be exploited as biofertilizers to sunflower under water-limited conditions.

Inoculation with drought-tolerant PGPR to crops can be a tool to induce drought tolerance in cereals leading to sustainable production goals. Further, rhizobia having EPS, sugars, and protective protein-producing ability and antioxidant activity can help plants to survive under stress and improve the efficiency of nutrient (Tilak 2004; Walley et al. 2007) and water use (Miller et al. 2003; Lenssen et al. 2007).

Salinity Stress

Certain rhizobial isolates are capable to survive under saline conditions, and their distribution in the rhizosphere varies with their tolerance ability (Tate 1995; Naz et al. 2009; Sgroy et al. 2009). However, salinity tolerance may vary due to the growth habit of rhizobial strains. *Rhizobium* species grow fast, they are more salt tolerant, whereas *Bradyrhizobium* species are comparatively less salt tolerant due to slow growth rate (Elsheikh and Wood 1995). The strains from genus *Sinorhizobium* are more salinity tolerant than the strains belonging to *Rhizobium* and/or *Mesorhizobium* (Rejili et al. 2012). On the other hand, it has been reported by Amarger et al. (1997) that salinity tolerance is a strain-specific property rather than species specific. Moreover, salinity tolerance limit of rhizobia has been reported up to 100 mM NaCl and higher levels (Yelton et al. 1983; Zahir et al.

2010b; Laranjo and Oliveira 2011). Salinity tolerance in rhizobia is a complex phenomenon, and many regulatory mechanisms get started to safeguard the cell from damage (Zahran 1999). One of them includes intracellular accumulation of osmolytes (low molecular weight solutes) like trehalose, polyamines, glycine betaine, glutamate, and ionic potassium. *S. meliloti* has been reported to accumulate glycine betaine under stress through uptake or synthesis or both (Boscari et al. 2002).

Several studies have indicated improvement in crop growth and productivity with the inoculation of salinity-tolerant rhizobia which also minimizes the production costs and environmental hazards (Hartmond et al. 1987; Dixon et al. 1993). In a study conducted by Bano and Fatima (2009), alleviation of salinity stress in maize was observed due to the combined inoculation of *Rhizobium* with *Pseudomonas*. They recorded increases in the uptake of Ca, P, and K in maize; however, Na accumulation remained unaffected. Inoculation also increased the proline and protein content in maize but decreased leakage of electrolytes. Similarly, Hafeez et al. (1987) recorded significant improvement in growth and development of *Vigna radiata* due to inoculation with salt-tolerant rhizobia under salinity. Similarly, a higher number of nodules and an increase in nodule dry weight of mung bean were observed by Bouhmouch et al. (2005) due to *Rhizobium* strain RP119 inoculation at 340 mM NaCl salinity. Rhizobial isolates with indoleacetic acid (IAA)-producing ability have also demonstrated improvement in host plants under osmotic stress conditions (Malhotra and Srivastava 2006; Spaepen et al. 2007). Comparison between two strains of *S. meliloti*, 1021 and RD64, for inducing salinity tolerance at 0.3 M NaCl salinity revealed that the *M. truncatula* plants inoculated with RD64 showed lower expression of ethylene-related genes and improved nitrogen fixation and shoot dry weight and showed less symptoms of senescence over 1,021 inoculated plants (Bianco and Defez 2009). Zahir et al. (2010b) inoculated lentil with *Rhizobium phaseoli* strain N20 which was efficient in auxin production and was salt tolerant. They observed increase in the growth and yield of the crop under salinity due to inoculation, whether the precursor of auxin (L-tryptophan) was present or not. However, the combined application of *Rhizobium* N20 and L-tryptophan gave the highest increases in plant height, nodule numbers, plant biomass, grain yield, and grain nitrogen up to 28, 71, 61, 65, and 22 %, respectively, over uninoculated control treatment. After a series of experiments, Abdel-Salam et al. (2011) suggested that *Rhizobium* species have great potential for inducing salinity tolerance and increasing production/nodulation of legumes in arid and semiarid areas of the world. Moreover, Soliman et al. (2012) revealed that the combined inoculation of *Sinorhizobium terangae* and AM fungi mitigated the impact of salinity in *Acacia saligna* in sandy loam soil. They further explained that the combined inoculation increased plant vigor and growth rate at higher salinity; improved nodulation and chlorophyll, carbohydrate, and proline contents; increased N, P, K, and Ca contents; and reduced the Na uptake/accumulation.

Conclusively, rhizobial isolates capable to survive high salinity and having plant growth-promoting characteristics can be a better option to safeguard the crop plants from hazardous impacts of salinity. Moreover, the production of osmolytes, phytohormones, and stress-relieving enzymes would be helpful in rescuing the plants' defense system under salinity stress.

Temperature Stress

Rhizobia are highly sensitive to high temperature (Meghvansi 2006) because it hampers their growth, limits survival, and reduces root colonization capacity (Munevar and Wollum 1981). However, optimal temperature for their growth ranges from 25 to 31 °C (Harwani 2006), whereas most of the rhizobia cannot grow at 37 °C (Graham 1992). But high temperature tolerance ability varies from species to species and even from strain to strain (Laranjo and Oliveira 2011). Several studies have indicated the ability of rhizobia to survive at high

temperatures due to the production of heat-shock proteins (Vriezen et al. 2007) and accumulation of compatible solutes or protectants (Santos and da Costa 2002). Therefore, researchers tried to find out that the rhizobial isolates, which can survive and tolerate high temperatures, could be helpful to plants under heat stress upon inoculation. Rahmani et al. (2009) inoculated soybean with thermotolerant *Bradyrhizobium* strain and recorded significant increases in nitrogen fixation, nitrogen contents, shoot dry weight, and grain yield. They suggested that the isolates from geographically hot regions are the potential candidates to be used as inoculants under heat stress. Zahran (1999) has also recognized the selection of rhizobia on the basis of stress tolerance as a good criterion. Bianco and Defez (2009) inoculated heat-tolerant *S. meliloti* strain 1021 to *M. truncatula* and observed reduced senescence, more nitrogen fixation, less reduction in shoot dry weight, and reduced expression of ethylene signaling genes. However, the use of compatible rhizobial strains along with intrinsically compatible cultivars suited to the local environment and rhizobia would be more appropriate (Shamseldin 2007). For this reason, Shamseldin (2007) conducted an experiment in Egyptian soil using indigenous rhizobia and common bean cultivar to measure the competitive ability of *R. etli* (strains EBRI 1 and EBRI 26) to occupy nodules as compared to gus-labeled *R. tropici* CIAT 899G. Results from the study described 78 and 62.5 % more nodule occupancy by EBRI 1 and EBRI 26, respectively, over strain CIAT 899G at 35 °C. Similarly, Kulkarni and Nautiyal (1999) inoculated *Prosopis juliflora* with five rhizobial strains NBRI12, NBRI133, NBRI329, NBRI330, and NBRI332 (tolerant to high temperature stress) and observed 30–89 % improvement in dry weight with respect to uninoculated control.

Thus it can be assumed that the rhizobial strains efficient in their survivability under high temperature, competent in root/nodule colonization, and belonging to the indigenous environment could be suitable inoculants in ameliorating the impact of high temperature stress in plants.

Heavy Metal Stress

Unlike organic pollutants, metals cannot be degraded/mineralized, but they can be transformed from toxic to nontoxic form. Therefore, microorganisms have evolved intrinsic ability to reduce or detoxify heavy metals through various mechanisms. These mechanisms may include biosorption, bioaugmentation, biostimulation, metal reduction, and metal ion efflux from the cell (Outten et al. 2000). For this reason, using heavy metal detoxifying or reducing microbes is an economical and preferred approach for reducing stress impact on plant growth and bioremediation technologies. Keeping this in view, Gupta et al. (2004) reported PGPB as heavy metal stress-reducing agents. Similarly, enhanced phytoremediation efficiency has been reported by the inoculation of metal-resistant and nitrogen-fixing bacteria (Khan et al. 2009). Bacteria have also the capability to adsorb or desorb toxic heavy metals like Zn and Ni to reduce their toxic effect on plants (Mamaril et al. 1997). *R. leguminosarum* bv. *viciae* isolated from the root nodules of faba bean has been reported as an efficient biosorbent for Cd^{2+} and Co^{2+}. The strain was highly tolerant to heavy metal stress in descending order, i.e., Ba^{2+}, 80 ppm; Zn^{2+}, 70 ppm; Co^{2+}, 50 ppm; Al^{3+}, 40 ppm; Ni^{2+}, 30 ppm; and Cd^{2+}, 10 ppm (Abd-Alla et al. 2012). Increased dry matter and improved symbiosis in peas were observed due to the inoculation of *Rhizobium* in Cd-contaminated soils (Ausili et al. 2010). Panigrahi and Randhawa (2010) alleviated the impact of As toxicity in alfalfa through the inoculation of *S. meliloti* strain 1021, and they observed higher concentration of As in the shoots of inoculated plants. Similarly, Dary et al. (2010) reported 4–10 times increase in metal accumulation and more biomass and nitrogen content in *Lupinus luteus* plants inoculated with *Bradyrhizobium* sp. in a multimetal-contaminated soil. They suggested that the inoculation improved phytostabilization potential of *L. luteus* in multi-metal-contaminated soil. Fan et al. (2011) inoculated *Medicago lupulina* with Cu^{2+}-tolerant *S. meliloti* CCNWSX0020 and recorded increase in biomass up to 46 and 78 %, respectively, in a growth medium

containing 0 and 100 mg Cu^{2+} kg^{-1}. Inoculation with *S. meliloti* also increased the Cu^{2+} contents in plant tissues up to 39 % where Cu^{2+} was supplied at 100 mg kg^{-1}. Finally, they suggested that the symbiotic relationship between *S. meliloti* CCNWSX0020 and *M. lupulina* can be utilized for the bioremediation of Cu^{2+}-contaminated soil. Rhizobia in effective symbiosis improve the soil fertility with nitrogen fixation and act as bioremediator for As in contaminated soils (Mandal et al. 2008). In an experiment conducted by Wani et al. (2008a), lentil was inoculated with Zn-tolerant *Rhizobium* RL9 under Zn contamination at 4,890 mg kg^{-1}. They measured increases in the grain protein, grain yield, nodule number, nodular dry mass, leghemoglobin, and plant dry matter up to 8, 10, 15, 27, 30, and 150 %, respectively. In another study, Wani et al. (2008b) demonstrated that the Cr contamination in soil at 136 mg kg^{-1} caused toxicity to chickpea plant, but the inoculation with Cr-reducing *Mesorhizobium* strain RC3 improved the grain protein, seed yield, dry matter, and nodule number up to 16, 36, 71, and 86 %, respectively, as compared to uninoculated control.

Biotic Stress

Plants suffer from several bacterial, fungal, or viral diseases during their life cycle where PGPB may help crop plants to overcome the situation through antibiosis, antagonism, competition, and ISR. Rhizobia have been reported as effective biocontrol agents in crop plants. They produce secondary metabolites in the form of antibiotics (Robleto et al. 1998), chitinases (Kacem et al. 2009), HCN (Ahemad and Khan 2009), and β-1-3-glucanase and proteases (Compant et al. 2005). Rhizobia produce bacteriocins and proteinaceous compounds to kill closely related pathogenic bacteria (Hafeez et al. 2005). Although the bacteriocins are narrow-spectrum antibacterial compounds, they are very effective against bacterial plant pathogens (Cladera–Olivera et al. 2006). Kacem et al. (2009) recorded the highest potential of *Rhizobium* species strains ORN 24 and ORN 83 for controlling the olive knot disease causing agent *Pseudomonas savastanoi* by the production of bacteriocins. Similarly, *R. leguminosarum* bv. *trifolii* has been reported to produce high-spectrum antibiotic peptide trifolitoxin against plant pathogens (Triplett et al. 1994; Breil et al. 1996). Certain rhizobia are efficient siderophore producers and their affinity to sequester Fe^{3+} varies tremendously (Ahemad and Khan 2010). Siderophore-producing ability makes rhizobia competent inhabitants in the rhizosphere where they outcompete the pathogenic microorganisms. Arora et al. (2001) reported that iron-deprived pathogens (*M. phaseolina*) die due to iron deficiency in the presence of siderophore-producing rhizobia. In the same way, Deshwal et al. (2003) evaluated the siderophore-producing *Bradyrhizobium* strains AHR 2, AHR 5, and AHR 6 against *M. phaseolina* which caused charcoal rot disease in peanut. The isolates inhibited the growth of *M. phaseolina* and reduced its population in peanut rhizosphere. Vargas et al. (2009) showed varied antagonistic potential of *R. leguminosarum* bv. *trifolii* against fungus *Verticillium* species, and they suggested that the variation was due to the difference in the type of siderophores produced by rhizobia. In addition to an increased iron availability in the rhizosphere due to siderophore activity, plants and microbes can also be saved from Al^{3+} toxicity. *R. leguminosarum* bv. *viciae* alleviates Al^{3+} toxicity by the production of hydroxamate siderophore vicibactin (Rogers et al. 2001). However, chitinase produced by rhizobia degrades the cell wall of pathogenic fungi. Rhizobia belonging to *R. leguminosarum*, *B. japonicum*, and *S. meliloti* have been reported to kill pathogenic fungi (*Rhizoctonia*, *Macrophomina*, and *Fusarium*) by dissolving their cell walls (Ehteshamul–Haque and Ghaffar 1993; Ozkoc and Deliveli 2001). In the same way, Hossain and Martensson (2008) also observed dissolution of fungal mycelium at the initial growth stage in the presence of chitinase-producing rhizobia. Moreover, rhizobia have been documented as elicitors or inducers of resistance against disease/pathogens in crop plants (Elbadry et al. 2006). They co-inoculated

R. leguminosarum bv. *viciae* FBG 05 with *P. fluorescens* FB 11 and recorded significant reduction in the incidence of bean yellow mosaic potyvirus (BYMV) disease in faba bean. Plants have multicomponent defense systems including structural barriers, hypersensitive response, and chemical weapons which are activated at varied extent with the inoculation of PGPB (Dixon et al. 1994). Rhizobial inoculation in chickpea activated the defense against *Fusarium oxysporum* which ultimately leads to the accumulation of phytoalexins and stimulated chalcone synthase, ammonia lyase, and isoflavone reductase enzymes (Arfaoui et al. 2007).

Rhizobia as Plant Growth-Promoting Bacteria (*Rhizobium*: Nonlegumes Association)

Rhizobia have been reported as symbionts of legumes where they fix atmospheric nitrogen and synthesize certain PGRs for the improved growth and productivity of plant. However, few researchers have demonstrated their ability to live in association with nonlegumes where they could be beneficial and harmful or have no effect on plant growth (Prayitno et al. 1999; Mehboob et al. 2011). But those who benefit plant growth while living in the rhizosphere/rhizoplane of the plant are termed as PGPR (Vessey 2003). However, these PGPR account up to 2–5 % of total bacterial population in the rhizosphere (Kloepper and Schroth 1978). For mutual association, both the partners contribute to have that relationship; therefore, plants exude substances (40 % of the plant dry matter) including amino acids, vitamins, organic acids, enzymes, nucleotides, sugars, and other plant metabolites in the rhizosphere (Rovera 1965; Raaijmakers et al. 2009). Associated microbes use these exudates as nutrient source and perform plant growth-promoting activities. Naher et al. (2009) observed a significantly higher population of *Rhizobium* species in the rhizosphere of rice where exudates produced by the rice roots included sucrose, arabinose, mannose, and xylose. Hence, it can be said that inoculated rhizobia/PGPR need to be associated with the plant roots with substantial population size so that they can improve plant growth by improving nutrient availability and rescuing hormonal and osmotic gradients by the production of osmolytes, PGRs, and stress-regulating enzymes. Induction of stress tolerance in plants, suppression of pathogenic fungi and bacteria in the rhizosphere by the production of chitinase and antibiotics, and competition for iron due to the production of siderophores are also the attributes of PGPB. Improvement in germination, root architecture, seedling vigor, shoot growth, stomatal conductance, chlorophyll contents, leaf area, photosynthesis, protein contents, grain yield, harvest index, and tolerance to abiotic stresses has been observed in nonlegumes due to the inoculation of rhizobia (Hilali et al. 2001; Hafeez et al. 2004; Hossain and Mårtensson 2008), whereas certain researchers have recorded plant growth inhibition in nonlegumes due to the overproduction of IAA (Antoun et al. 1998), HCN production (O'Sullivan and O'Gara 1992), auxin, and nitrates (Perrine-Walker et al. 2007) and bacteriocin-like compound production (Perrine-Walker et al. 2009) by inoculated rhizobia. The association between rhizobia and nonlegumes varies with respect to ecological conditions, soil type, indigenous microflora, cultural conditions, inoculated bacterial species, and plant variety (Depret et al. 2004; Mehboob et al. 2008; Hussain et al. 2009).

Rhizobia have associative relationship with nonlegumes, but that association can be loose or strong. They can associate to non-nodulating plants in three different ways depending on the place of the plant body. If they live in the rhizosphere, inside plant tissues, or upper plant parts, then their association is termed as rhizosphere, endophytic, or phyllospheric, respectively (Weyens et al. 2009; Mehboob et al. 2012). Colonization and survival potential of rhizobia with the roots of nonlegumes are comparable to their colonization capability in host legumes (Bhattacharjee et al. 2008). Many scientists have reported rhizobial colonization ability in different nonlegume crops including wheat (Afzal and Bano 2008), rice (Senthilkumar et al. 2009), maize (Cassan et al. 2009), cotton (Hafeez et al.

2004), potato (Sturz and Kimpinski 1999), tobacco (Ji et al. 2010), and lettuce (Noel et al. 1996). Nodule-like structures (hypertrophies) have been reported on the roots of nonlegumes due to rhizobia, but there was no evidence for atmospheric nitrogen fixation (Naidu et al. 2004). Shimshick and Hebert (1979) calculated 8×10^9 number of binding sites of rhizobia per gram fresh root (gfr) of wheat and rice at 22 °C by using the Langmuir adsorption isotherm. Similarly, Chabot et al. (1996) observed 4.1 and 3.7 CFU/gfr on maize and lettuce roots after 4 and 5 weeks of seeding. Rhizobial colonization of the root cortex, root meristem, and xylem of wheat was demonstrated by Sabry et al. (1997). In the same way, Chi et al. (2005) recorded the colonization of rhizobia from the rhizoplane to the stem base, leaf sheath, and leaves of rice plants.

Rhizobia are remarkable colonizers of non-nodulating plant roots, and during their stay in the rhizosphere, they utilize root exudates as carbon source and perform different plant growth-promoting activities to benefit plant growth. Hence, it can be concluded that the rhizobia and plants have an association where they contribute their parts to continue that beneficial mutual association.

Precursors (Phytohormone): Inoculum (Rhizobial) Interaction for Enhancing Crop Production

It is a well-established fact that rhizobial inoculation of legumes/nonlegumes (with suitable and efficient strains of rhizobia) significantly increases the yield of crops. Use of biological inoculants based on rhizobia/PGPR appears to be a promising supplement to chemicals, and biological inoculants are used worldwide for improving the growth and yield of inoculated legumes/cereals. Although inoculation of crop plants with efficient rhizobial/rhizobacterial strains significantly improves the growth and yield of legumes/nonlegumes and various researchers have reported 5–20 % increase in yield of different crops by these inoculants, there are certain limitations in this technology, especially the inconsistent results in field conditions due to a number of soil and environmental conditions. To improve the effectiveness of the inoculum and enhance reproducibility, Frankenberger and Poth (1987) developed the idea of precursor-inoculum interaction based upon the hypothesis that inoculation of PGPB in the presence of specific physiological precursor of a PGR is often more effective in promoting plant growth than inoculation alone. Several published studies have demonstrated the success of precursor-inoculum interactions on nonlegumes; however, scanty information of this approach is available on legumes. This area needs intensive research in depth and detail.

Tryptophan is considered to be the most suitable precursor of auxins in plant species and soil microorganisms (Khalid et al. 2006; Sridevi and Mallaiah 2008). Upon their addition to soil, an increase in auxin contents and its influence on plant growth and yield has been observed and correlated to microbial-derived auxins (Frankenberger and Arshad 1995; Zahir et al. 2005, 2010a, b).

Frankenberger and Poth (1987) conducted greenhouse experiments to study the effect of an auxin-producing ectomycorrhizae, *Pisolithus tinctorius*, and L-TRP on the growth of Douglas fir. Results revealed that the growth of Douglas fir was stimulated by *P. tinctorius* inoculation in supplementation with lower concentrations of L-TRP (10^{-8} to 10^{-6} M) most probably due to the interaction of soil indigenous microbiota with L-TRP. Hussain et al. (1995) used precursor-inoculum interaction for improving lentil yields. They observed that *Rhizobium* inoculation alone increased the grain yield by 21 % over uninoculated control; however, when combined with L-TRP (1.7 mg kg^{-1}), the *Rhizobium* inoculation further increased the grain yield by 31 % over the control. However, they further reported that auxin production by rhizobial inoculation increased by increasing L-TRP levels and incubation period. Anjum et al. (2009) studied precursor-inoculum interactions and reported that although rhizobia produced auxin without L-TRP supplementation, its addition to the medium increased auxin biosynthesis many folds under axenic conditions. They further reported that rhizobial inoculation increased mung bean seedling growth and

correlated to microbial-derived auxins in a pouch experiment under axenic conditions. Khalid et al. (2004) conducted trials on auxin biosynthesis in rhizosphere vs. non-rhizosphere soil and reported higher auxin production with selected PGPB in the rhizosphere soils compared to uninoculated soil. They concluded that inoculation with suitable strains and/or amendment with L-TRP could promote auxin biosynthesis in the rhizosphere soils ultimately resulting in better plant/crop growth and development.

Similarly, Zahir et al. (2010a) studied precursor-inoculum interactions and reported that although the separate application of rhizobia and/or L-TRP improved the plant growth and yield, their combined effect was more pronounced in improving growth and yield of mung bean (*V. radiata* L.) under field conditions. *Rhizobium* inoculation supplemented with L-TRP (10^{-4} M) gave the most promising results and significantly increased the nodule number and biomass plant^{-1}, grain yield, and 1,000-grain weight significantly as compared to uninoculated control. In another study, Zahir and co-workers (2010b) evaluated the performance of a rhizobial isolate N20 in the presence and absence of L-TRP for improving the growth and yield of mung bean under saline conditions. Upon their combined application to seed/soil, an increased influence on plant growth and yield was observed and correlated to microbial-derived auxins produced in the rhizosphere that reduced the adverse effects of salinity. They further concluded that supplementing *Rhizobium* inoculation with L-TRP could be a useful approach for improving the growth and yield of mung bean. Very recently, Qureshi et al. (2013) evaluated the potential of *Rhizobium* to enhance growth and fodder yields of maize in the presence and absence of L-TRP. The interaction of L-TRP and rhizobial species (Cp3, Br3, and Lt2) increased the fresh fodder and dry matter yield than their separate application. Likewise, Qureshi and his co-workers (2012) assessed the co-inoculation effect of *Rhizobium* and P-solubilizing *Bacillus* sp. in the presence of L-TRP. They observed that co-inoculation improved the pod and straw yield considerably, but the effect was more pronounced with L-TRP. They finally concluded that precursor-inoculum interaction is an efficient approach; however, it should be studied more comprehensively in different ecological zones to sustain the crop yield.

Overall, the published literature revealed the effectiveness of precursor-inoculum interactions than their separate application for improving the growth and yield of legume/nonlegume crops. However, further research is needed to unlock several horizons like physiochemical transformation of PGRs in the soil, screening efficient and inexpensive precursors of PGRs, and agronomic practices which could enhance the stability and bioavailability of PGRs in plant root zone and to develop an effective approach based upon precursor-inoculum interaction for the prosperity of agriculture industry.

Rhizobium-Based Commercially Available Inoculants

The application of PGPB for improving crop production is becoming an emerging technology owing to their environment-friendly traits. To take advantage of the demonstrated beneficial effects of various soil microbial groups in increasing plant growth and yields, many types of microbial inoculants (biofertilizers) have been in use for a long time. Biofertilizers are biological preparations containing live or latent cells of microorganisms or their metabolites, which when inoculated to seed, soil, or roots of seedlings promote plant growth and enhance harvestable yield. Biofertilizers, generally marketed, contain microbes capable of N_2 fixation, phosphate solubilization/mineralization, phytohormone production, and biocontrol activities. Rhizobial strains that are often used (singly or in mixture) include a range of genera: *Allorhizobium*, *Azorhizobium*, *Bradyrhizobium*, *Mesorhizobium*, *Rhizobium*, and *Sinorhizobium*. Species of other genera are also used but somewhat less frequently. Rhizobia-based inoculants have been used to improve legume nodulation and yield of legumes/nonlegumes, while bacteria belonging to *Azotobacter* and *Azospirillum* have been applied

Table 11.4 Commercially available *Rhizobium*-based biofertilizers

Bacteria	Product	Company
Rhizobia	VAULT® HP plus INTEGRAL®	Becker Underwood Corporate, USA
Delftia acidovorans and *Bradyrhizobium*	BioBoost	BrettYoung Seeds Ltd., Canada
Rhizobium sp.	SeedQuest®	Soygro (Pty) Ltd., South Africa
Rhizobium sp.	Legumefix	Legume Technology Ltd., UK
Bacillus subtilis and *Bradyrhizobium Japonicum*	HiStick N/T, Turbo-N	Becker Underwood Corporate, USA
B. subtilis and *B. japonicum*	Patrol N/T	United Agri Products (UAP) Inc., Canada
Burkholderia cepacia type Wisconsin	Deny	Market VI LLC, Vern Illum 6613 Naskins Shawnee KS 66216, USA
Rhizobium spp.	*Fasloon Ka Jarasimi Teeka*	AARI, Faisalabad, Pakistan
Rhizobium spp.	BioPower	NIBGE, Faisalabad, Pakistan
Rhizobium spp.	Biozote	NARC, Islamabad, Pakistan
Rhizobium spp. and PGPR	Rhizogold	ISES, UAF, Faisalabad, Pakistan

to enhance cereal growth (as biofertilizers), and mainly *Bacillus* and *Pseudomonas* have been applied for biocontrol (as biopesticides) of plant diseases (Fravel 2005; Bravo et al. 2011). Table 11.4 shows some selected commercially available rhizobial inoculants with their producers/trade name. The development of techniques for the large-scale production of pure inoculants, with high infectivity potential, is the main issue to be tackled in order to allow a wide use of biofertilizers. The key aspects in rhizobial inoculation (biofertilizers) technology are the use of a proper formulation of inoculant preparations, the selection of an adequate carrier, and the design of correct delivery methods.

Conclusions and Future Perspectives

The conventional approach to increase agricultural productivity through massive inputs of chemical fertilizers/pesticides is not sustainable because of high costs and concerns about global warming, environmental pollution, and safety. The search for microbes that improve soil health and enhance plant nutrition has continued to attract attention due to the increasing cost of fertilizers and their negative environmental effects. For a more comprehensive development and utilization of rhizobial inoculants, there are several issues/recommendations which need to be taken care of in future research.

- The repertoire of rhizobial effects and functions mentioned in the review clearly suggests that they would be promising to increase crop yield, remove contaminants, inhibit pathogens, and produce fixed nitrogen or novel substances if we manage effective communities to favor plant colonization by rhizobial bacteria.
- It also demonstrates the possibility of reducing chemical fertilizers with biofertilizers, which in turn will help in mitigating the major global problem of environmental pollution and also fetch premium in the agriculture market.
- In the regions where legumes have no response to inoculation with rhizobia, co-inoculant PGPR may be an alternative for the enhancement of crop production.
- Co-inoculation with other PGPR is an efficient strategy that would have better prospects to act synergistically with rhizobia for better productivity of legumes compared to simple inoculation under sustainable agriculture system.
- Moreover, the commercial inoculant products are beginning to emerge that could prove useful in a commercial agriculture setting.
- Exploitation of multifunctional microbial inoculants seems feasible to enable plants to behave in a more competitive way and to

survive when established under stress conditions/environments.

- Comprehensive studies on the analysis of nodulation kinetics are needed which could enable to investigate the role of specific substances as signals or molecular components involved in the nodulation process.
- PGPB may communicate with the plant by means of various mechanisms to augment their growth and interfere with each other when used in consortium. However, actual mechanisms involved during consortium interactions still remain to be elucidated to harness the real potential of multi-strain inoculants.
- Studies on characterization of the interaction between sets of PGPR and rhizobia under various ecological soil environments and the changes in metabolic activities caused by co-inoculants affecting plant performance are also needed to be researched for achieving commercially realistic and effective co-inoculants.

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Legume-Rhizobia Symbiosis Under Stress

12

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Abstract

Legume-rhizobia symbioses are of practical importance in provision of sustained food supply. Currently, it provides approximately 45 % of N used in agriculture. This quantity will have to be augmented as the world's population increases and natural resources used in production of fertilizer-N diminish. The major constraints for food security are soil salinity, sodicity, nutrient deficiency, and toxicity which are driven by indiscriminate use of agrochemicals including chemical fertilizers and pesticides, utilization of excess water resource coupled with climate changes, and periodic droughts. In this scenario, legume-rhizobia symbioses are nonpolluting, cost-effective ways to enhance high N_2-fixing potential under stress conditions. Several symbiotic systems that are tolerant in extreme conditions of salinity, alkalinity, acidity, drought, toxic doses of fertilizer, and metal toxicity have been identified including rhizobial symbiosis in arid region. Extreme environmental conditions adversely affect rhizobia-legume interactions wherein rhizobial partner utilizes endogenous or exogenous osmolytes and secretes specific proteins to alleviate the problem of aridity, salinity, and toxicity. Thus, change in rhizobial population can be an indicator of soil fertility. Hence, osmoadapted rhizobial strains can be used as biofertilizers for salt-sensitive crops in saline soils. In this context, interaction among rhizobia, plant growth-promoting rhizobacteria (PGPR), and mycorrhiza are also important. Here, we give an account of relevance of biological nitrogen fixation (BNF) in sustained food supply, effects of extreme conditions on legume-rhizobia symbiosis, as well as interaction

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of rhizobia with belowground microbial diversity including mycorrhiza and adaptive strategies of rhizobia under condition of stress. We also discuss about models wherein osmotolerant rhizobia can be used as biofertilizers and sustain green revolution to evergreen revolution.

Introduction

The major challenge for agricultural industry is to provide sustained food supply for nearly eight to nine billion people, when 40 % of the world's agricultural land is affected by salinity-, drought-, sodicity-, and toxicity-related problems. The intensive agricultural practices like cultivation of short duration crops which require large amount of water, excessive application of fertilizers, and indiscriminate use of pesticides and herbicides that played a key role in bringing about green revolution are major causes of soil quality degradation. The harsh soil conditions adversely affect legume-rhizobia symbiosis. The importance of legume-rhizobia symbiosis in agricultural and forestry ecosystems cannot be underestimated. It fixes about 45×10^6 metric tons of nitrogen annually and provides nearly half of all the nitrogen used in agriculture (Brockwell et al. 1995). To maintain sustained food supply, the quantity of nitrogen needed for agriculture is projected to double by year 2030 (Tilman 1999). BNF also sequesters carbon. Biologically fixed nitrogen at current estimate, 45×10^6 metric tons of nitrogen per year, is equivalent to sequestering an additional 770 to 990×10^6 metric tons of carbon year^{-1} (Vance 2001; Abberton 2010). Thus, legume-rhizobia symbiosis that is environmentally prudent in N_2 fixation will benefit both agriculture and the environment. Reactive oxygen species (ROS) can adversely affect the survival of rhizobial cells during desiccation, when the water content drops below 40 %. Arid regions are characterized by high temperature, low relative humidity, high evaporation, and scanty rainfall, all leading to salinity. Drought can also lead to hyperosmotic stress, accumulation of salts and solute, impairment of metabolism when water activity has reduced, and formation of ROS.

Agrochemical Stress

Natural resources are not sufficient to fulfill food requirement of the growing population. Hence, various agrochemicals are being used to enhance crop production and protection. Agrochemicals like pesticides are very important in crop protection from various pests. The excessive use of pesticides over the years has, however, caused environmental problems such as accumulation of agrochemicals in soil and groundwater. Out of total agrochemicals applied to a crop, only 15 % reach the target. Their fate in soil system depends upon factors such as soil sorption, leaching, volatilization, surface runoff, and their uptake by plant system. Agrochemicals present in soil affect micro- and macroflora including bacteria, actinomycetes, fungi, nematodes, arthropods, crustaceans, earthworms, and leguminous plants. These soil flora play an important role in the degradation of organic matter as well as in nitrification, nitrogen fixation, and release of various nutrients from soil (Pandey and Singh 2004; Seghers et al. 2003). Thus, the use of agrochemicals has become a major threat to beneficial soil microbes and affects the sustainability of agricultural crops. Agrochemicals also interact with microorganisms that reside in the rhizosphere like rhizobia and reduce the number of available root sites for infection (Anderson et al. 2004). Furthermore, agrochemicals block the signaling mechanisms between rhizobia and leguminous plants and inhibit the interaction between them (Fox et al. 2007).

Rhizobia: Diversity and Occurrence in Soil

Rhizobial population in soil is often diverse because of biotic as well as abiotic factors. Genotypes of host plants and their distribution

affect the occurrence and distribution of rhizobial species in soil. For example, *Phaseolus vulgaris* and *Mimosa affinis* differ in their nodulation specificity for legumes. Whereas, *P. vulgaris* is rather nonspecific for its microsymbiotic nodulating partner and is nodulated by six rhizobial species, viz., *Rhizobium etli*, *R. giardinii*, *R. gallicum*, *R. tropici*, *R. leguminosarum* bv. *phaseoli*, and *Bradyrhizobium* spp.; on the other hand, *M. affinis* is rather specific and is nodulated with *R. etli* alone (Wang et al. 1999). Genistoid legumes (brooms) in Canary Islands, Morocco, and Spain are nodulated by *Bradyrhizobium* strains that belong to four distinct evolutionary lineages that include *B. japonicum*, *B. canariense*, and two unnamed genospecies (Vineusa et al. 2005). Abiotic factors like pH, rainfall, soil type, and temperature affect the diversity of rhizobial species, while soil types may also affect the composition of rhizobial community. This is very well exemplified by a legume wherein a host growing in different geographical locations is nodulated by different rhizobial species/genera. For example, *Glycine max* (common name soybean) is usually nodulated by *B. japonicum* globally; however, in the Xinjiang region of China, *Mesorhizobium tianshanense* and *Sinorhizobium fredii* have been recovered from this legume. In another example, *R. leguminosarum* bv. *viciae* and bv. *trifolii* have been reported in bean nodules growing in Leon, France, whereas in addition to these two rhizobia, *R. etli*, *R. gallicum*, and *S. fredii* have been reported in beans growing in Andalucia region (Velázquez et al. 2001). Traditionally two rhizobial species (*Mesorhizobium ciceri* and *Mesorhizobium mediterranean*) have been reported from nodules of *Cicer arietinum*, but *Ensifer meliloti* (formerly *Sinorhizobium meliloti*) was reported from nodules of this legume growing in Tunisia under water-deficient conditions (Romdhane et al. 2009). Similarly, *E. meliloti* has also been identified in *C. arietinum* plants growing in Almora and Terai region of Uttarakhand Himalayas (Rajwar et al. 2013). The presumption that rhizobial diversity is affected by soil type is strengthened by identification of different rhizobial species from Caragana plant growing in three ecoregions of China varying in soil types. *Mesorhizobium* genospecies I, II, IV, VI, and VII were reported from Caragana plants growing in sandy soils of Mongolia. *M. temperatum*, *M. tianshanense*, *M. septentrionale*, *M.* genospecies III, *R. yanglingense*, and *Rhizobium* sp. IV were reported in those growing in saline/alkaline soils and *M. plurifarium*, *M.* genospecies V and VII, and *Rhizobium* sp. IV in fertile/forest soils of Northwestern Yunnan region (Lu et al. 2009).

Stress Responses in Plant and Rhizobia

There are stress responses in both plant and rhizobia. The following sections discuss stress responses and adaptive mechanisms in plants and rhizobia separately and how various osmolytes help to form an effective symbiosis and maintain good productivity under stress.

Stress Responses in Plants

Drought

Drought is an environmental condition that produces water stress in plant (Kozlowski and Pallardy 1997). The primary mechanism which plants adopt to grow in drought is regulation of water loss. Water loss in plants is regulated either by morphological modifications or physiological adaptations. Morphological modifications include thickening of cuticle and development of deep root systems. Physiological adaptations include leaf rolling, responsive stomata, metabolism at low leaf water status, and phytohormone secretion (Turner et al. 2000). Thus, plants with water potential less than that of soil survive (Tiaz and Zeiger 2002).

Salinity

Soil salinity leads to accumulation of high concentration of sodium (Na^+), chloride (Cl^-), and sulfate (SO_4^{2-}) ions in plant cells, inactivating enzymes and inhibiting protein synthesis. This reduces photosynthesis and in turn plant growth (Shannon 1997). Salinity-adaptive responses in plants include accumulation of compatible solutes that raise the osmotic pressure in cytoplasm,

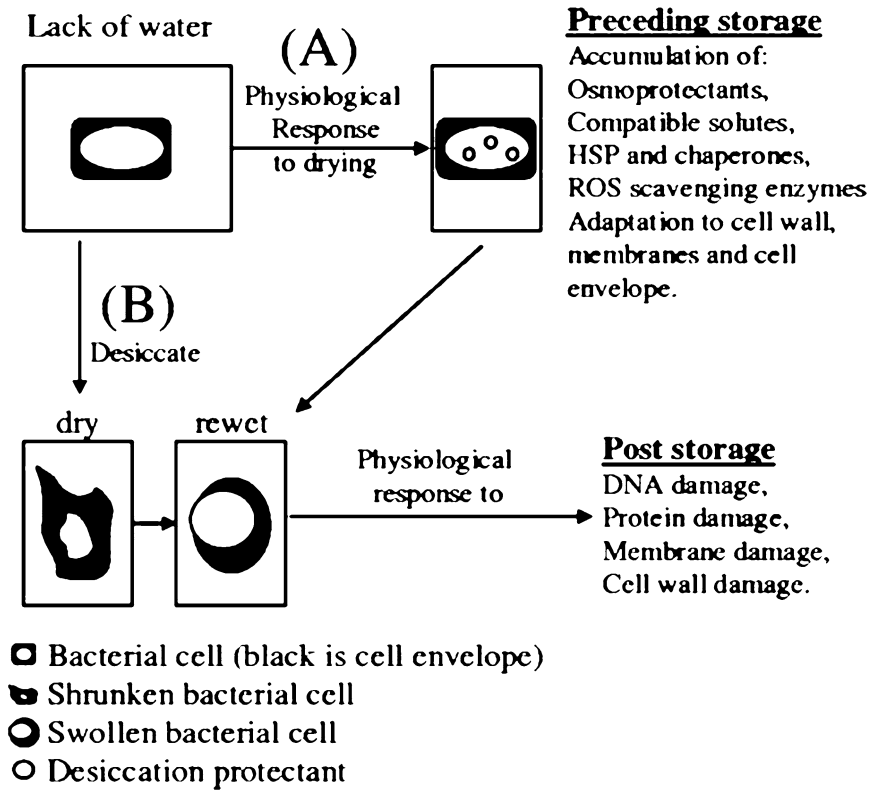

Fig. 12.1 Pathway highlighting response of rhizobia under desiccation and salt stress

stabilize protein and membrane (McNeil et al. 1999), and increase in sodium ion uptake (Hasegawa et al. 2000). The plant can synthesize various compatible solutes, i.e., betaines tertiary sulfonium compounds, polyols, sugars (e.g., trehalose), and amino acids (e.g., proline).

Stress Responses in Rhizobia

Rhizobial species vary in their intrinsic osmotolerance which is measured in terms of their ability to grow in varying concentration of NaCl. *B. japonicum*, *R. etli*, and *R. leguminosarum* (all three biovars) are salt sensitive with their growth inhibited completely at 100 mM NaCl (Boncompagni et al. 1999); *Mesorhizobium huakuii*, *R. tropici* IIB, and *S. fredii* are moderately sensitive with their growth inhibited at 200 mM NaCl, whereas *S. meliloti* and *Agrobacterium tumefaciens* are highly salt tolerant with the ability to grow at 300 mM NaCl (Bernard et al. 1986). *Rhizobium* spp. from nodules of *Acacia*, *Hedysarum*, *Leucaena*, and *Prosopis* plants can tolerate up to 500 mM NaCl (Zhang et al. 1991). Rhizobia survive in stress due to expression of NaCl-responsive loci leading to the accumulation of osmoprotectants, enhanced production of exopolysaccharides, ROS-scavenging enzymes, and heat shock proteins and chaperons (Vriezen et al. 2007). Vriezen and coworkers have proposed two hypothetical pathways for rhizobial response under desiccation and salt stress (Fig. 12.1).

Compatible Solutes

Rhizobial cells are affected by changes in osmolarity of their surroundings. They are plasmolyzed in saline and arid habitat and adapt to changes in osmolarity by accumulation of compatible solutes or osmolytes that confer protection against the deleterious effects of the low water activity, maintain appropriate cell volume,

and protect intracellular macromolecules (Le Rudulier et al. 1984). The uptake of compatible solutes is preferred over synthesis. Compatible solutes are of various types: amino acids and related structures (glycine betaine, proline betaine, ectoine, glutamate, and dimethylsulfoniopropionate), carbohydrates (sucrose, trehalose, maltose, and cellobiose), alcohols (glycerol, mannitol), amino acids (pipecolate), and potassium. The type and amount of compatible solutes that accumulate in rhizobia depend on the level of osmotic stress and growth phase of culture (Smith et al. 1994). Osmolytes are either synthesized de novo or from precursors acquired from external environments (Galinski 1995). Osmolytes such as trehalose, sucrose, potassium, and glutamate are synthesized de novo (Miller and Wood 1996; Welsh 2000). In *S. meliloti*, glutamate is accumulated at low and high salt concentrations; but at higher levels besides glutamate, N-acetyl glutaminyl glutamine amide (NAGGN) is also accumulated (Smith et al. 1994). Varying types of compatible solute accumulate within rhizobial cell viz-á-viz growth phases under stress. Glutamate and NAGGN are accumulated at late exponential phase and trehalose at stationary phase (Talibart et al. 1997).

Compatible solute glycine betaine is synthesized from choline through its oxidation. Choline is widespread in soil and rhizosphere, as its derivative phosphatidylcholine is a component of eukaryotic membrane (Boncompagni et al. 1999). Glycine betaine confers osmotolerance to cells grown in inhibitory concentration of salts as well as during lag and early exponential phases. β-dimethylsulfoniopropionate and α-pipecolic acids are accumulated in *Sinorhizobium* spp. (Pichereau et al. 1998; Gouffi et al. 2000).

Role of Exopolysaccharides

Exopolysaccharides (EPS) are involved in maintaining successful symbiotic interaction (Broughton et al. 2000). EPS are involved in several steps of nodule development like invasion, release of bacteria from infection thread, and differentiation of bacteroid (Fraysse et al. 2003). In stress, EPS play a major role in the survival of microorganisms (Rinaudo 2004). They help in two ways: exclusion of toxic compounds, such as chloride (Cl^-) (Lloret et al. 1998), and their ability to form a diffusion barrier to oxygen (Potts 1994), thus leading to higher survival of rhizobia. There are two types of EPS: EPS I (succinoglycan) and EPS II (galactoglycan). EPS I is further divided into low-molecular weight (LMW) and high-molecular weight (HMW) types. EPS I and II are helpful in mitigating the harmful effect of ROS (Lehman and Long 2013), which is enhanced under drought stress (Jebara et al. 2010).

Role of ROS-Scavenging Enzymes

ROS-scavenging enzymes are accumulated during desiccation-induced salinity stress in interacting partners of successful legume-rhizobia symbiosis. Leguminous plants are able to detoxify ROS by two mechanisms: nonenzymatic and enzymatic. Nonenzymatic is through accumulation of flavanones, carotenoids, and ascorbic acid, whereas enzymatic involves superoxide dismutases, peroxidases, and catalases. In bacteroids, peroxidases are not produced; hence, an alternative ascorbate-glutathione (ASC-GSH) cycle enzymes become operative (Jebara et al. 2010). It has earlier been reported that peat-based rhizobial formulations show increased accumulation of manganese-based superoxide dismutase (Feng et al. 2002).

Regulation of Stress Responses in Rhizobia

Water-stressed soils are low in nitrogen and phosphorous. The production of EPS I under nitrogen-limiting conditions involves three genes: *exo*Q, *exo*T, and *exo*P (Gonzalez et al. 1998; Skorupska et al. 2006). ExoQ (product of *exo*Q gene) is responsible for the production of HMW succinoglycan and exoT for LMW succinoglycan, whereas ExoP1 and ExoP2 (the product of *exo*P genes) block the polymerization of HMW succinoglycan and hence promote production of LMW succinoglycan. The production of ExoP is regulated by a two-component signal transduction system in which ExoS and ChvI act as sensor kinase (Cheng and Walker 1998) and SyrM and SyrA as response regulator (Mulligan and Long 1989). Thus, it can be concluded that the expression

of genes involved in EPSI production is upregulated during salt stress (Ruberg et al. 2003). During osmotic stress induced by the abundance of solutes out of two, the production of HMW succinoglycan is more than LMW succinoglycan (Breedveld et al. 1990). Under drought stress, available phosphorous in the soil is low (0.1–10 μM). This stimulates the production of EPS II (Mendrygal and Gonzalez 2000; Schmeisser et al. 2009). The production of EPS I is regulated by proteins of the phosphotransferase (PTS) system (Pinedo et al. 2008). The genes involved are hpr (for substrate), hprK (for phosphorylation), and EIIA (manX) (for catabolite repression) and are located immediately downstream of the exoS gene (Pinedo and Gage 2009). Phosphorylation/dephosphorylation of its substrate (HPr) is controlled by hprK genes through succinate-mediated catabolite repression mechanism. A phosphorylated form of HPr activates EIIA and enhances catabolite repression. Out of three genes of PTS system, hpr gene is responsible for the production of EPS I and EIIA for accumulation of EPS I. The strains that have mutated EIIA and hpr genes form an effective nodulation on *Medicago* spp. (Pinedo et al. 2008).

The synthesis of EPS II is regulated by two proteins: WggR (ExpG) and MucR (Baumgarth et al. 2005). WggR, a product of the gene located in the galactoglucan synthesis gene cluster, positively affects the synthesis of EPS II under phosphorous limitation (Becker et al. 1998), whereas MucR represses galactoglucan synthesis (Mendrygal and Gonzalez 2000). Under P starvation, WggR and phosphorylated PhoB bind to the promoter regions and activate the transcription of a few other *wga*, *wgd*, and *wge* genes (Bahlawne et al. 2008). PhoB activates transcription of *wggR*.

Bacterial motility is also an important factor for the establishment of symbiosis under stress conditions. The regulatory systems controlling the production of EPS also control the motility of rhizobia (Bahlawane et al. 2008). The motility genes are regulated along with chemotaxis and flagellar genes and placed into class I, II, and III. Class I genes comprise the *visN* and *visR* genes. Class I genes control Class II genes, which comprise the flagellar assembly and motor genes. Class III genes include flagellin and chemotaxis genes (Sourjik et al. 2000). The regulatory system ExoR/ExoS/ChvI, which is involved in the regulation of EPS I synthesis, affects motility genes. It was reported that rhizobial strains with mutations in *exoR* and *exoS* lose their ability to swim and swarm as the expressions of all three classes of flagellar biosynthesis genes were downregulated (Yao et al. 2004).

Under oxygenic stress, four genes *katA*, *katB*, *katC*, and *sodA* are expressed. The gene *katA* is induced by H_2O_2, while *katC* is induced by heat, salt, ethanol, and paraquat. Thus *katC* is important for alleviation of ROS. In leguminous plants during osmotic stress, induced by low water activity or ionic radical, an NaCl-responsive locus becomes active. It shuts down photosynthetic apparatus, and hence ROS production stops (Vriezen et al. 2007). *S. meliloti* can survive desiccation as it has a genetic mechanism induced by NaCl. The NaCl-responsive locus is induced mainly due to osmotic stress either by reduction of water activity or accumulation of ionic radicals. Two NaCl-responsive genes *asnO* and *ngg* increase the ability of rhizobial strains to grow in high NaCl concentrations. The product of these genes (AsnO and Ngg) is involved in the production of a compatible solute NAGGN by a two-step regulatory mechanism where Ngg (N-acetyl glutaminyl glutamine synthetase) converts glutamine to a dipeptide N-acetyl glutaminyl glutamine (NAGG), and AsnO transfers the amide nitrogen from glutamine to N-acetyl glutaminyl glutamine to produce NAGGN (Sagot et al. 2010). *S. meliloti* strain are not able to grow optimally at higher NaCl concentration if anyone of the two genes of NaCl-responsive locus is not expressed (Vriezen et al. 2013).

Legume-Rhizobium Symbiosis Under Stress

Structural and Biochemical Changes Under Water Stress

Drought- and high salinity-related water stress has negative effect on the nodulation ability of leguminous plants (Rao and Sharma, 1995). If at all nodulation occurs, there is a reduction in

nodule size, increase in rate of nodule senescence, and thus BNF is less (Babber et al. 2000). Structural changes in nodules of leguminous plants grown in salinity-affected soils have been observed. These are lobed nucleus with modified chromatin distribution, endoplasmic reticulum, microtubules, mitochondria, and plastids (Serraj et al. 1995). Several metabolic changes are also observed during stress-induced senescence in nodules. These are increased concentration of sucrose due to inhibition of enzyme sucrose synthase (Ben Salah et al. 2009; Sanchez et al. 2011), decrease in malate concentration leading to reduced bacteroid respiration (Prell and Poole. 2006; Marino et al. 2007), and decrease in ascorbic acid content that has a free radical-quenching ability. Effective utilization of enzyme phosphoenolpyruvate (PEP) carboxylase maintains structural integrity of nodule in stress (Drevon et al. 1998; Galvez et al. 2005). Drought leads to decrease in leghemoglobin content (Gordon et al. 1997). Although an antioxidant enzyme catalase is present, but peroxidase is absent; thus ascorbate-glutathione cycle enzymes serve as an alternative peroxidase-scavenging system in nodules (Jebara et al. 2010).

Sheokand et al. (1995) observed that catalase activity in nodules of *C. arietinum* and *Cajanus cajan* growing in the presence of 100 mM NaCl concentration decreases to 44 %. Vadez and Sinclair (2002) reported higher accumulation of ureides in drought-sensitive lines of soybean as compared to tolerant lines and that high accumulation of ureide in nodule decreases nitrogen fixation (King and Prucell 2005). Lodwig et al. (2003) suggested that a complex amino acid cycling occurs in nodules. Plants provide amino acids to the bacteroids enabling them to shut down their ammonium assimilation through BNF. Some non-nitrogenous signals are also involved in the inhibition of BNF under drought. These are oxidative signals created by ROS. ROS can affect the expression of sucrose synthase at transcriptional and posttranslational levels (Marino et al. 2008). It is now recognized that redox signals play a crucial role in establishing and maintaining symbiosis between rhizobia and legumes (Chang et al. 2009). The natural stress hormone like abscisic acid (ABA) concentration was increased fivefold in soybean nodule under drought stress (Clement et al. 2008). Although ABA is not involved in the decline of sucrose synthase, though it might affect the leghemoglobin-facilitated O_2 diffusion and BNF (Gonzalez et al. 2001).

Proteomics: A Tool for Understanding Legume-Rhizobia Symbiosis Under Stress

Several genes that are present in micro symbionts during normal conditions but expressed under conditions of stress have been identified as early as 1998, when techniques for proteome analysis were still not being frequently used (Zurayk et al. 1998). It has already been revealed that rhizobia adapt to stress at physiological (by producing osmolytes and EPS) and enzymatic level (ROS-scavenging enzymes). The genes for their production are present, but expressed under specific condition. Proteome analysis may give an insight into key plant and microbial proteins expressed during the interaction of legume with rhizobia under stress only. Since gene analysis reveals little about their expression, proteomics have become an important tool for studying the biology of legume-rhizobia symbiosis in real time. Djordjevic (2004) has characterized proteins specifically produced in nodules when *S. meliloti* nodulates *Melilotus truncatula* and *Melilotus alba* under stress. Proteins identified are chaperonic, those involved in detoxification of ROS and osmoregulation. Chaperonin proteins, GroEL and GroES, and Hsp are upregulated under stress (Yeh et al. 2002). ABC transporter proteins that are responsible for sodium ion (Na^+) transport are downregulated under stress and hence prevent cell rupturing (Shamseldin et al. 2006). During early colonization of rhizobia, cell receptors (like Myc factors) recognize stigmasterols released by plants and induce calcium spiking within cell which is responsible for activation of calmodulin-like proteins within the nucleus that will further activate the nodulation-specific genes of microsymbiont (reviewed by Rajwar et al. 2013). Under drought stress, these calmodulin-like proteins CaML2 and CaML4 get downregulated, thereby inhibiting nodulation (Estibaliz et al. 2007).

Integrating proteomics, genomics, transcriptomics, and metabolomics studies with bioinformatics and statistical tools may help identify genes and proteins which govern legume-rhizobia interactions under stress. These studies will help design crop plants that are better suited to interact with microbes. It is proposed that better understanding of beneficial and detrimental interactions between the plants and microbes may offer unprecedented opportunities to increase crop productivity.

S. meliloti-Legume Symbiosis Under Stress: A Case Study

M. ciceri are natural symbionts of *C. arietinum* (Nour et al. 1994). But *Sinorhizobium* spp. have been reported in the same legume growing under drought stress in Tunisia (Romdhane et al. 2009), Almora, and Terai region of Uttarakhand, India (Rajwar et al. 2013). This indicates that *Sinorhizobium* spp. have stress adaptation mechanism. In a study carried out in our laboratory at the Department of Microbiology, G.B. P. U. A & T., Pantnagar, the amount of EPS produced by *S. meliloti* strain MTCC 11403 increased from 146 to 234 μg ml^{-1} under drought stress. We also found that the same strain when subjected to water stress colonize *C. arietinum* rhizosphere (7.65×10^6 cfu/g of root) more than the rhizosphere of normal watered plant (1.25×10^5 cfu/g of root) (Karmakar, unpublished). In *S. meliloti* strains, EPS I of both types: HMW and LMW are produced. During osmotic stress induced by abundance of solutes, HMW succinoglycan is produced more than LMW succinoglycan (Breedveld et al. 1990). Thus, it can be concluded that expression of genes involved in EPSI production is upregulated during salt stress (Ruberg et al. 2003). Under drought stress, available phosphorous in the soil is low (0.1–10 μM). This stimulates the production of EPS II (Mendrygal and Gonzalez 2000; Schmeisser et al. 2009). This may be the reason for *Sinorhizobium* to occupy the nodule under drought stress. The mechanism of EPS production in *Sinorhizobium* was elucidated by Skorupska et al. (2006). They reported that two regulatory systems, one with three genes, *exo*Q, *exo*T, and *exo*P, and another with ExoS and ChvI, are involved. Similarly under phosphorous-limiting condition, PhoB and ExpG get upregulated, which collectively enhance the production of EPS II. The study of Davies and Walker (2007) revealed that EPS-producing genes (*exo*P, *exo*Q, and *exo*D) are assisting in nodulation because strains mutated in any one of the EPS-producing genes were unable to nodulate *Medicago*.

Legume-Rhizobium Symbiosis Under Agrochemical Stress

Pesticides are agrochemicals that protect agricultural crops from damaging influences such as weeds, insects, or phytopathogens. While pesticides are used for crop protection, they ultimately reach soil system and influence microbes, other soil communities, as well as plants. A few pesticides can affect rhizobial communities in soil and legume-rhizobia symbiosis (reviewed by Ahemad and Khan 2013). Pesticides affect legume-rhizobia symbiosis in several ways. These affects are summarised in Table 12.1. One of them is reduction of root biomass because fewer sites are available for rhizobial infection: this adversely affects the supply of carbohydrates to the existing nodules with resultant decrease in the potentiality of rhizobia for infection. As a consequence, meristematic activity decreases that is otherwise essential for nodule development (Anderson et al. 2004).

Other is blocking of molecular communication between flavonoids of plants and nod proteins of rhizobia thus preventing nodulation. Rhizobia and pesticides compete for common binding site(s) on plant roots. For example, glyphosate [N-(phosphonomethyl) glycine] is a nonselective herbicide and competes for binding site(s) on plant roots and moves to the plants through phloem. Glyphosate acts upon shikimate acid pathway; blocks synthesis of aromatic amino acids and other phenolic compounds such as cinnamic acids (Canal et al. 1987), flavones (Ishikura et al. 1986), and flavonoids (Laanset 1987); and eventually decreases plant growth. The action of glyphosate decreases synthesis of starch (Greiger and Bestman 1990), causes imbalance in hormone

Table 12.1 Commonly used pesticides and their effects on rhizobia

SN	Name of pesticide	Effect of pesticides
1.	Metribuzin (H), Glyphosate (H), Thiamethoxam (I)	Decrease production of indole-3-acetic acid and siderophore
2.	Thiaram (F)	Decrease population of *Rhizobium* and *Mesorhizobium*
3.	Mancozeb (F)	Reduce the growth by alteration in the membrane composition
4.	Chlorpyrifos (one), Lindane(I)	Inhibit the growth of *Rhizobium japonicum*
5.	Captan(F)	Inhibit the growth of *Rhizobium*, *Mesorhizobium*, *Sinorhizobium*, *Bradyrhizobium*
6.	2,4-D(H)	Produce deleterious effect by modifying the membrane fluidity
7.	Hexaconazole(F)	Inhibit the dehydrogenase system in *Rhizobium*
8.	Atrazine (H)	Decrease the nodules number

H herbicides, *F* fungicides, *I* insecticides

levels (Lee 1984), reduces synthesis of chlorophyll (Kitchen et al. 1981a) and its precursor δ-aminolevulinic acid (ALA) (Kitchen et al. 1981b), and reduces the activity of nitrogenase enzyme complex (Malik and Tesfai 1985). Herbicides inhibit photosynthesis and indirectly reduce the amount of total nitrogen fixed by legumes. Metribuzin and fluchloralin inhibit transfer of electrons from compound Q to plastoquinone in photosystem II preventing $NADP^+$ reduction that is required for CO_2 fixation (Fedtke 1982). However, the activity of nitrogenase enzyme remains unaffected in the presence of herbicides. Dexon p-(dimethylamino) benzenediazo sodium sulfonate a fungicide commonly used to control "root rot" or "damping off" in legume seedling leads to nodulation failure due to interferences in plant hormones, indole acetic acid, and gibberellic acid. Herbicides negatively affect legume-rhizobia interaction reducing the production of surface polysaccharides (LPS and EPS) of rhizobia.

Effect of Macronutrient-Containing Agrochemicals on Rhizobia-Legume Interaction

Nitrogen is the most critical nutrient for plant growth. Plant yield increases when compatible rhizobia are inoculated along with fertilizers in fields (Elsheikh 1992). Leguminous plants fix nitrogen with the help of nitrogen-fixing bacteria and remove the problem of nitrogen limitation (Rasanen and Lindstrom 2003). Nitrogen concentration in soil also regulates the formation of nodules (Rhoades and Coleman 1999). Phosphorus, on the other hand, is essential for synthesis of nucleic acids, phospholipids and ATP, protein synthesis, and N_2 fixation (Buchanan et al. 2000). Available phosphorous increases the number of nodules formed in legume roots (Rasanen and Lindstrom 2003). Potassium activates numerous enzymes and acts as a counterion and cellular osmoticum (Epstein and Bloom 2005). It affects the growth rate of internodes (de la Gardia and Benlloch 1980). The growth of *R. leguminosarum* bv. *trifolii* and *S. meliloti* is restricted in medium which is low or deficient in potassium. Calcium spiking is necessary for efficient nodulation in the root hair (Wais et al. 2002). The role of calcium redistribution in the infection process is also reported (Downie and Walker 1999; Niebel et al. 1999).

Tripartite Relationship Under Drought/Salinity Stress

Presently arid or semiarid land areas are increasing worldwide because soil is becoming saline on account of intensive irrigation in the agricultural fields and rising temperatures, thus limiting crop production. Although plants have their own mechanisms to cope up with drought stress, they become more tolerant to drought when associated with different soil microorganisms such as rhizobia, arbuscular mycorrhizal fungi (AMF), and other PGPRs. Interaction among these microorganisms enhances plant drought tolerance at physiological and molecular levels through

decreased oxidative stress, improved water status, or regulation of aquaporins. There is a correlation between drought tolerance in rhizobia and rhizobia-induced plant drought tolerance. Under drought conditions, PGPRs regulate the levels of stress-related hormones, i.e., abscisic acid and ethylene. The regulation of these hormones is the cause of an enhancement of plant drought tolerance mediated by PGPR. At the same time, AMF in combination with rhizobia or PGPR usually has an accumulative beneficial effect on plant drought tolerance (Aroca and Lozano 2009).

For sustainable crop production, it is necessary to improve salt stress tolerance in crops. Thus, development of salt-tolerant crop plants through plant breeding programs is one approach, but this may be available for a few plant species. Alternatively, seeds and seedlings of leguminous crops can be inoculated with various PGPR(s) to alleviate effects of salt stress in crop plants (Dixon et al. 1993; Tank and Saraf 2010). It has been indicated that PGPR strains produce polysaccharides that bind to sodium ions (Na^+) in root zone and hence alleviate effect of salt stress on plant and enhance microbial growth and activities (Tank and Saraf 2010).

Salinity affects rhizobia-legume symbiosis by direct impairment of early interactions during nodule formation and nodule development (El-Hamdaoui et al. 2003). Moreover, symbiotic N_2 fixation in legumes is a phosphorous-requiring process, and salinity significantly reduces plant nutrient uptake, especially phosphorus, since phosphate ions are precipitated under salt-stressed conditions (Grattan and Grieve 1999). It has been shown that some PGPRs can increase P availability to plants by solubilizing insoluble phosphates; this improves P nutrition through increased BNF as well as availability of other nutrients to plants (Gyaneshwar et al. 2002).

There are reports of unsuccessful nodulation under salt stress. It may be due to failure in the infection process because of the adverse effect of salinity on the establishment of rhizobia in rhizosphere of leguminous plant (Singleton and Bohlool 1984; Elsheikh and Wood 1990). The legume-rhizobia symbiosis and the process of nodule initiation on legumes are sensitive to salt stress (Lauter et al. 1981; Rai and Prasad 1983; Velagaleti and Marsh 1989). Salinity enhances ethylene production which causes nodule senescence (Glick 2004; Glick et al. 2007; Madhaiyan and Poonguzhali. 2007). Under high salinity, most of the available energy is used in osmotic adjustments by the plant (Munns and Termaat 1986) and thus growth is affected. Under macronutrient deficiencies, especially nitrogen and phosphorous, the increase in ethylene levels in roots is observed (Lynch and Brown 1997). Borch et al. (1999) reported that P-deficient roots of common bean produced twice as much ethylene based on root dry weight than P-sufficient roots. The increased ethylene production in roots causes reduction in nodulation under low P conditions caused by salinity. It has been reported that increase in root-shoot biomass ratio under salt stress is an index of salt tolerance. An increased functional root biomass has been shown to strengthen the root Na^+ detoxification capability (Hua et al. 1982). It seems that the increased ethylene production in salt-stressed roots may contribute towards reduction in root growth compared with shoot growth.

PGPRs have also evolved a variety of adaptive mechanisms in order to restore the cell turgor pressure and reduce the osmotic potential between cell and environment, subsequently maintaining cell division and cell elongation under salt stress conditions. Among these mechanisms, one response of PGPR is the transient adjustment in ionic balance (Egamberdiyeva 2007). Sodium and chloride ions are regarded as being biologically aggressive osmolytes (Zhu 2002). High concentrations of these ions in the plant accordingly lead to imbalances in water and ion relations. Giri et al. (2003) reported that accumulation of Na^+ was strongly influenced by storage of other cations, particularly potassium ions. Increased K^+ concentration under saline conditions may help to decrease Na^+ uptake, and this can indirectly maintain the growth of plant (Giri and Mukerji 2004). Co-inoculation of *Rhizobium*

and *Pseudomonas* significantly increased the K^+ content of plants under salt stress conditions.

Mycorrhiza is relatively more important to plant growth under dry conditions than when soil moisture is plentiful (Michelsen and Rosendahl 1990). Plant infected by AMF can tolerate and recover more rapidly from soil water deficit than uninfected plants (Henderson and Davies 1990). Root hairs are very efficient in increasing the root surface area, although their effect on uptake may not be proportional because of competition among them. AM develop symbiotic associations with plants, and their hyphae are more efficient than root hairs and provide better transport of minerals from the soil to the root over much larger distances than by diffusion.

Alleviation of the Impact of Stress

Medicago sativa, *Arachis hypogea*, *Cyamopsis tetragonoloba*, and *Melilotus* spp. are major crop species, and *Acacia* and *Prosopis* are important leguminous trees growing in arid regions. The osmoadaptation of both the partners is essential for maintaining successful symbiosis and nitrogen fixation in these regions. Rhizobia can nodulate their host even under stress conditions.

Drought Stress

Jenkins et al. (1987) identified rhizobia that effectively nodulate arid legumes. Wadisirisuk et al. (1989) observed that osmotolerant rhizobia can migrate even under scarce moisture condition. Athar and Johnson (1996) reported that osmotolerant strains of *R. meliloti* (now *S. meliloti*) performed better than those of the non-tolerant alfalfa rhizobia and formed effective symbiotic relationship under drought. Peoples et al. (1998) compared the annual herbage production under stress and reported that *Medicago-based* pastures could fix more than 70 kg of Nha^{-1} $year^{-1}$, making them efficient and sustainable. Straub et al. (1997) reported that seed yield in plants inoculated with proline mutant (pro^-) rhizobia decrease twice than those inoculated with pro^+ strains. Thus, rhizobial strains with ability for increased accumulation of compatible solutes are able to perform better in stressed soil. Another approach to improve legume-rhizobia symbiosis is to isolate rhizobia from wild legumes and inoculate them in cultivated legumes. Zhao et al. (1997), working on this approach, isolated *Rhizobium* strains from *Astragalus* spp. and *Cicer* spp. and successfully inoculated them into *M. sativa* and *P. vulgaris*.

Trees of genera *Acacia* and *Prosopis* are of central importance in rural economy (Roshetko 2001). Hocking (1993) reported that these trees can grow in areas with rainfall as low as 40–50 mm $year^{-1}$. During rainless period, long tap roots of trees reach the groundwater (Deans et al. 1999). Fagg and Stewart (1994) reported the contribution of these trees towards soil stabilization and improvement through nitrogen fixation. Masutha et al. (1997) evaluated their agroforestry potential, nodulation ability, and nitrogen fixation potential. Kadiata et al. (1996) reported that nitrogen fixed by different tree legumes were: *Leucaena* (Indian subabul), 20–74 %; *Albizia*, 28–72 %; and *Cliridia*, 44–84 %. Even under water stress prevailing in these regions, they could fix 43–581 kg of nitrogen per hectare as compared to annual legumes which fix 15–210 kg of nitrogen per hectare (Dakora and Keya 1997).

Salt Stress

Root nodule-colonizing bacteria are more salt tolerant than their legume host and show variation in salt tolerance (Zurayk et al. 1998). The growth of many rhizobial species is inhibited by 100 mM NaCl (Yelton et al. 1983); however, Mohammad et al. (1991) have reported that a few can tolerate NaCl concentration of 300–700 mM. Breedveld et al. (1991) reported that *R. leguminosarum* can tolerate up to 350 mM NaCl. The fast-growing strains of soybean and chickpea rhizobia can also tolerate up to 340 mM NaCl compared to slow-growing strains (Elsheikh and Wood 1995). Mashhady et al. (1998) reported that *S. meliloti* formed successful symbiosis with *M. sativa* under saline condition, i.e., 100 mM

NaCl. Among tree species, *Prosopis articulata*, *P. pallida*, and *P. tamarugo* can grow and fix nitrogen under 300 mM NaCl concentration (Felker et al. 1981). Salt-tolerant Australian *Acacia* (*A. auriculiformis* and *A. stenophylla*) can survive even up to 1.7–1.8 M NaCl (Aswathappa et al. 1987).

Conclusion

In stressed soils, signaling between legumes and rhizobia which is important for development of successful legume-rhizobia interaction is negatively affected. Signaling involves three key steps: (1) flavonoid is released by plants to activate the *nod* D genes of rhizobia, (2) activated nod D gene upregulates nod boxes and form nod factors, (3) and endogenous plant hormones are activated by nod factors and mediate nodule initiation. Individually legume and rhizobia possess mechanisms to overcome stress. But several biotic and abiotic factors influence legume-rhizobia symbiosis under stress. Therefore, for the establishment of successful legume-rhizobia symbiosis, the following strategies are proposed.:

Selection of Competitive Strains of Rhizobia

Rhizobial strains with the ability to colonize rhizosphere competitively (Howieson et al. 1995), catabolize secondary metabolites present in root exudates (Boivin et al. 1991), synthesize osmolytes under stress de novo, and produce high EPS under water and nutrient limitation must be selected by screening natural populations. The performance of selected strains under stress can be further enhanced by genetically modifying the strains for traits like rhizosphere competence, rhizopine catabolism, and toxin production. For example, introduction of NolA (a repressor of *nod* genes) prevents colonization of legume roots by native bacteria (Vlassak and Vanderleyden 1997). Rhizobia with *moc* genes have rhizopine-catabolizing ability which enhances their nodule occupancy (Murphy et al. 1995). Incorporation of toxin-producing genes enhances bactericidal effect of inoculant rhizobia over native populations. EPS-producing genes like *exoQ*, *exoT*, and *exoP* may be overexpressed constitutively, and NaCl-responsive genes transferred to develop effective legume-rhizobia symbiosis in stress environment.

Breeding for Competitive Legumes

Breeding programs should target quantitative trait loci (QTL) controlling morphological traits like nodule number per plant and nodule weight (Neo et al. 1996). Legume traits such as high shoot nitrogen content, nitrate tolerance, antioxidant enzymes, and hormones can be also targeted.

Biofertilizers Augmented with Elicitor Molecules

In the current scenario of mass production of biofertilizers, it is not only the quality that is essential but also its specificity towards alleviation of stressed conditions. Towards this end, newer technologies are being employed using various strategies. In such an attempt, signal molecules characterized from root exudates of leguminous plants are being added to the end product. The molecules being used in this refined technology include flavonoids, sugars, acids, amino acids, amines, and secondary metabolites that function as elicitor or signaling molecules for successful microbe-plant interaction (Skorupska et al. 2010). As discussed earlier, all these signal molecules are usually altered during stress conditions like drought and salinity. Currently, companies like Nitragin are marketing formulations with biosignals that improve the symbiotic relationship, activate mechanisms to resist abiotic stress conditions, and induce pathogen-defensive response in plants (www.nitragin.com.ar).

Thus, selecting competitive rhizobial strains, breeding for competitive legume host, and using

elicitor-loaded biofertilizers can be useful in sustaining green revolution to evergreen revolution.

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Legume Root Exudates: Their Role in Symbiotic Interactions

13

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Abstract

Legumes are in constant dynamic interactions with rhizobia and arbuscular mycorrhizal (AM) fungi which mutually benefit the partners. Legumes provide the carbon energy to the microbes, and they in turn provide the nutrients such as nitrogen and phosphorus. The growth of these symbionts largely depends on the secretions of the legume roots which include both high and low molecular weight compounds. These molecules also act as cues in plant-microbe signaling and recognition. A cascade of reactions take place between the legume and the microbe before specific refined symbiotic partnership manifests mutually benefiting both the partners. Here, we provide an overview of the functions of legume root exudates with emphasis on the interactions between legume and rhizobia.

Introduction

The root system, often referred to as the "hidden" part of the plant, not only provides anchorage and facilitates nutrients and water uptake to the plant but also excretes numerous chemical compounds which act as mediators of the plants' various interactions in the rhizosphere. The secreted exudates include sugars, polysaccharides, amino acids, aromatic acids, aliphatic acids, fatty acids, sterols, phenolics, enzymes, proteins, plant growth regulators, and secondary metabolites (Badri et al. 2009). These exudates result in complex biological and chemical interactions in the rhizosphere including beneficial interactions of the plant with rhizobia, mycorrhizal fungi, and plant growth-promoting rhizobacteria (PGPR) or negative or defensive interactions with parasitic plants, pathogenic bacteria or fungi, and invertebrate herbivory. The plant root and its various interactions result in the creation of unique conditions in the rhizosphere which is different compared to the bulk soil (Benfey and Scheres 2000). However, secretions of these compounds come with a significant carbon cost to the plant (Marschner 1995), which could be as much as 30–40 % of fixed carbon in young seedlings (Whipps 1990). Root exudates also help regulate the microbial community in the rhizosphere.

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It has been observed that microbes tend to be more abundantly present in the rhizosphere (Hiltner 1904). The bulk of the organic carbon in the soil is contributed via root exudates of plants (Hutsch et al. 2000; Nguyen 2003). The aim of this review is to provide recent overview of the functions of legume root exudates in the legume-rhizobia and AM symbiosis with special emphasis on flavonoids and strigolactones.

Flavonoids and Strigolactones: Two Important Groups of Legume Root Exudates

Flavonoids are one of the most well-studied compounds in root exudates (Table 13.1). Flavonoids are key signaling compounds in the legume-rhizobia symbiotic interactions (Fisher and Long 1992) and AM symbiosis (Vierheilig et al. 1998; Vierheilig and Piche 2002). They are also known to be involved in the defensive interactions of the plant with pathogens (He et al. 1996; Wasmann and Etten 1996; Enkerli et al. 1998). Flavonoids are phenylpropanoid, biologically active low molecular weight secondary metabolites, mostly synthesized from p-coumaroyl-CoA and malonyl-CoA precursors (Stafford 1990). Flavonoids are a diverse group of compounds containing more than 10,000 members and encompassing flavones, flavonols, flavan-3-ols, flavanones, isoflavonoids, isoflavans, and pterocarpans (Ferrer et al. 2008). Their diversity is derived by the substitution of the flavonoid basal structure by glycosylation, malonylation, methylation, hydroxylation, acylation, prenylation, or polymerization (Winkel 2001). Among the flavonoids, isoflavonoids restricted primarily to the *Leguminosae* have been widely reported to be involved in the legume-rhizobia symbiotic interactions and defense against microbial pathogens. In the legume-rhizobia symbiosis, they act as inducers of a nodulation gene (Ferguson and Mathesius 2003) and also function as a precursor in the biosynthesis of phytoalexin during plant-pathogen/herbivore interactions (Aoki et al. 2000; Dastmalchi and Dhaubhadel 2014). Biosynthesis of isoflavonoids occurs through the general phenylpropanoid pathway. L-phenylalanine, the precursor of isoflavonoids, is first converted to cinnamate in a reaction catalyzed by the enzyme phenylalanine ammonia-lyase (PAL). Hydroxylation of cinnamate at the para position of the phenyl ring by cinnamate 4-hydroxylase then produces p-coumarate. The final step of the general phenylpropanoid pathway is completed with the production of CoA thiol esters by the 4-coumarate-CoA ligase. The first step in the flavonoid pathway is catalyzed by the enzyme chalcone synthase, which is the formation of a chalcone scaffold via the condensation of one CoA thiol ester and three malonyl-CoA molecules. Chalcone reductase then reduces the flavonoid intermediate to form deoxychalcone, which is in turn acted upon to produce the flavonoid core flavonone through cyclization by the enzyme chalcone isomerase. The reaction at this point then enters the legume-specific branch pathway to produce the isoflavone intermediate 2-hydroxyisoflavonone from flavonone by a novel aryl ring migration. The final products in the pathway such as the isoflavone genistein and daidzein are formed either through spontaneous dehydration of 2-hydroxyisoflavonone or through the catalytic reaction of 2-hydroxyisoflavonone dehydratase (Dixon et al. 1995; Wang 2010).

Legume roots also release strigolactones, a small class of carotenoid-derived compounds with only 15 members having been structurally characterized so far (Matusova et al. 2005; Alder et al. 2012). Strigolactones are known as key signaling molecules in the initial stage of the AM symbiosis (Besserer et al. 2006, 2008) and have also been recently reported to play a role in the legume-rhizobia symbiosis as positive regulators of nodulation (Soto et al. 2010; Foo and Davies 2011; Foo et al. 2013). Structurally, strigolactones are composed of four rings (A–D). The A and B rings show variation due to the different side groups, but the C and D rings owing to their role in the essential biological activity are highly conserved (Xi et al. 2010; Cheng et al. 2013). Interestingly, strigolactones and abscisic acid share a common carotenoid biosynthetic pathway but diverge at β-carotene (Matusova et al. 2005; Rani et al.

Table 13.1 Root exudates identified from legumes and their functions

Legume host plant	Rhizobial strain	Compound	Function	Reference
Medicago sativa	*Sinorhizobium meliloti/3654, 1683, 1354*	Luteolin and chrysoeriol	Nod gene and chemotaxis inducer	Hartwig et al. 1990
			Nematode repellent	
Trifolium repens	*Rhizobium leguminosarum* bv. trifolii	Umbelliferone	Inhibition of nod gene	Djordjevic et al. 1987
		Formononetin, 7,4′-dihydroxyflavone	Transcriptional activation of nod gene promoters	
Glycine max	*Bradyrhizobium japonicum/9105*	Daidzein	Induces nod gene, nematode repellent. Induces nod gene	Kosslak et al 1987; Bassam et al. 1988
		Genistein		
		Coumestrol		
Vigna unguiculata	*Rhizobium* sp. strain NGR234	Daidzein, genistein	Nod gene and chemotaxis inducer	Dakora 2000
		Coumestrol 7,4′-dihydroxyflavone		
Phaseolus vulgaris	*R. etli* CFN42/4381, 642, 605, 371, 250, 194, 184	Eriodictyol, naringenin	Induces nod gene	Hungria et al. 1991
		Genistein, 7-O-glycoside		
		Delphinidin		
		Petunidin		
		Malvidin		
		Myricetin		
		Quercetin		
		Kaempferol		
Vicia sativa	*R. leguminosarum* bv. Viciae/5057,870, 684, 488, 352, 151, 147	3,5,7,30-Tetrahydroxy-40-methoxyflavone, 7,30-dihydroxy-40-methoxyflavone	Induction of the nod genes	Zaat et al. 1989
Sesbania rostrata	*Azorhizobium caulinodans*/5369	7,40-Dihydroxyflavone	Induction of the nod genes	Messens et al. 1991
Medicago truncatula	*S. meliloti*	7,40-Dihydroxyflavone	Induction of the nod genes	Zhang et al. 2007
Robinia pseudoacacia	*Mesorhizobium* sp.	7,40-Dihydroxyflavone	Induction of the nod genes	Scheidemann and Wetzel 1997
		Apigenin		
		Naringenin		
		Chrysoeriol		
		Isoliquiritigenin		
Cicer arietinum	*R. ciceri*	Naringenin, hesperetin, naringin, 70H coumarin, luteolin	Induction of the nod genes	Srivastava et al. 1999
			Anti-inducer of the nod ABC genes of *R. ciceri*	

2008; Lopez-Raez et al. 2010). Consequently, abscisic acid has been assumed to have a regulatory effect on strigolactone biosynthesis (Lopez-Raez et al. 2010). Key enzymes involved in the synthesis of strigolactones are the carotenoid cleavage dioxygenases 7 and 8 (CCD7 and CCD8) and the iron-binding protein D27. Strigolactone levels in the root are also regulated by the levels of nitrogen and phosphorus in the soil. Depletion of nitrogen and phosphorus in the soil usually results in elevated levels of strigolactones in the roots; however, there is no positive correlation between the regulation of mycorrhizal symbiosis and nodulation by strigolactones with nutrient deficiency (Foo et al. 2013).

Root Exudates in the Legume-Rhizobia Symbiosis

Flavonoids: The Primary Signal Molecule of Legume Root Exudates

In addition to their diverse role in plants such as the regulation of auxin transport (Brown et al. 2001; Wasson et al. 2006), modulating levels of reactive oxygen species (Taylor and Grotewold 2005; Agati et al. 2012), and imparting colors to various tissues including flowers (Davies et al. 2012), flavonoids also play an essential role in the formation of the root nodule during the early stages of the legume-rhizobia symbiosis. Flavonoids act as chemoattractants to compatible rhizobia in the rhizosphere, near the root tips where the greatest amounts are released (Graham 1991). The sites of rhizobial infection, which are the emerging root hair zone, have been shown to have the optimal concentrations of flavonoids (Zuanazzi et al. 1998). The legume-rhizobia interaction leading to the formation of a symbiotic nitrogen-fixing nodule is a very specific interaction. In part, flavonoids are known to play an important role in influencing and determining this specificity. The expression of *Sinorhizobium meliloti nod* genes was inhibited by the soybean (*Glycine max*)-produced daidzein and genistein, while the same isoflavonoids induced the expression of *nod* genes in *Bradyrhizobium japonicum*. However, luteolin was found to induce the *S. meliloti nod* gene expression (Peters et al. 1986). NodD proteins, which are members of the LysR family of transcriptional regulators, are usually needed for the transcription of genes required for the synthesis of Nod factor. Activation of the transcription of Nod factor biosynthesis genes requires both NodD proteins and a flavonoid coinducer. Binding of NodD and an appropriate flavonoid to the *nod* boxes located at the promoter regions induces bending in DNA at the binding site which allows RNA polymerase to initiate gene transcription (Chen et al. 2005; Cooper 2007).

Flavonoids have also been reported to enhance the growth of rhizobia. Flavonoids such as coumestrol and daidzein extracted from soybean roots were shown to promote the growth of *Rhizobium japonicum* USDA 138 and *R. leguminosarum* (D'Arcy-Lameta and Jay 1987). Promotion of rhizobial growth by flavonoids could probably be due to the ability of rhizobia to both convert flavonoids to monocyclic aromatics such as protocatechuic acid and 4-hydroxybenzoate (Rao and Cooper 1994) and utilize the β-ketoadipate pathway to channel these products into the citric acid cycle (Harwood and Parales 1996; Parke 1997: Cooper 2007). In another mechanism employed by rhizobia, biotin released by *Medicago* is perceived by an *S. meliloti* regulatory locus *bioS* resulting in its enhanced growth and root colonization (Streit et al. 1996; Heinz et al. 1999). Further, flavonoids also influence the symbiotic efficiency, metabolism of rhizobia, and regulation of cell division during development of nodules (D'Arcy-Lameta and Jay 1987; Subramanian et al. 2007). Legume root exudates are also known to act as chemoattractants for rhizobia. Besides flavonoids, rhizobia also show positive chemotaxis to other compounds present in root exudates such as simple sugars, amino acids, and carboxylic and hydroxyaromatic acid (Cooper 2007).

Studies in our laboratory have found differential induction of proteins in *Mesorhizobium ciceri* strain DKA-1 when grown with the root exudates of four different genotypes of chickpea. The effect was concentration dependent as well as genotype dependent, clearly indicating the role of the chemical composition of the root exudates (Fig. 13.1). Chemical profiling of a large number of chickpea genotypes would help in identifying the metabolites which are conducive for effective symbiotic interactions as well as develop a diagnostic marker for segregating high and low nodulators and nitrogen fixers.

Strigolactones Involved in the Legume-Rhizobia Symbiosis

While the importance of strigolactones in mycorrhizal symbiosis is well established, their role in the legume-rhizobia symbiosis has only recently gathered interest (Steinkellner et al. 2007).

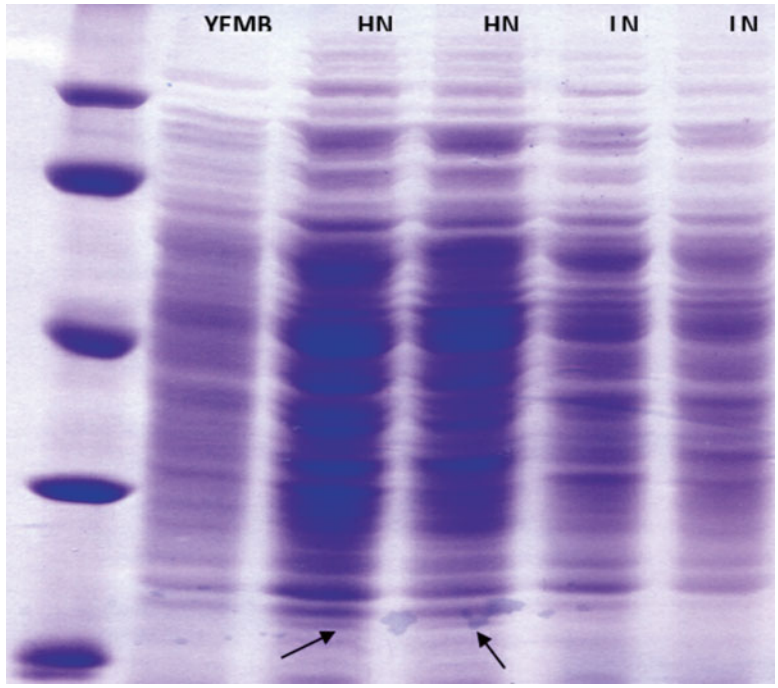

Fig. 13.1 SDS-PAGE profiling of *Mesorhizobium* sp. DKA-1. A 26kD protein was induced in DKA-1 grown with root exudates of high nodulating genotypes (HN). This was absent in DKA-1 grown in YEMB or with root exudates of low nodulating genotype (LN)

Application of synthetic strigolactone analogue Gr24 was associated with increase in the number of nodules formed in *Medicago sativa* and wild-type pea (Soto et al. 2010; Foo and Davies 2011). Contradictory observations were, however, reported in pea (Foo et al. 2013). Flavonoid-induced nodulation-specific rhizosphere signals such as enhanced growth, stimulation of *nod* factor production, or induction of calcium spiking were not detectable when Gr24 was inoculated directly to rhizobial cell cultures (Moscatiello et al. 2010; Soto et al. 2010). Foo et al. (2013) suggested that since strigolactones also play a pivotal role as an endogenous plant hormone in both root and shoot tissue, their role in promoting nodulation could also be as an endogenous plant signal within the root.

Nod Factors Are Induced by Flavonoids

Nod factors are key signal molecules produced by rhizobia in response to flavonoid secretion by legumes. They are lipo-chito-oligosaccharides (LCOs) with an oligomeric backbone of β-1, 4-linked *N*-acetyl-D-glucosaminyl residues, which are N-acylated at the nonreducing terminal residue (Denarie et al. 1996; Kamst et al. 1998). The number of GlcNAc residues present in the chito-oligosaccharide backbone, in the nature of the fatty acyl group, and in the substituents at the nonreducing and/or reducing terminal residues results in variation in the structure of Nod factors (D'Haeze and Holsters 2002). Expressions of nodulation (nod) genes (*nod*, *nol*, and *noe* genes) are required for Nod factor synthesis. Several physical, physiological, and molecular responses are elicited in plants at an active Nod factor concentration of $c.10^{-12}$ mol l^{-1}. These responses include root hair deformation; depolarization of plasma membrane; calcium spiking; alterations in the root hair cytoskeleton; formation of preinfection thread in deformed root hair; nodule primordial cortical cell division; reactive oxygen-generating system inhibition; perturbation of auxin flow in roots (in conjunction with flavonoids); and induction of nodulin gene expression at the preinfection, infection, nodule development, and nodule function stages of the

symbiosis (Bartsev et al. 2004; Cooper 2007). The expression of symbiosis-specific gene and subsequent nodule formation is initiated by the binding of specific Nod factors to the LysM receptor kinase (Perret et al. 2000; Antolin–Llovera et al. 2012). The oligosaccharide backbone of Nod factors can undergo various substitutions which influences the binding of Nod factors to the LysM receptor kinases (Perret et al. 2000).

Secreted Proteins Determine Host Specificity/Range

Proteins secreted by rhizobia are known to play important roles in determining host range in the legume-rhizobia interactions. At least three mechanisms of secretions of proteins by rhizobia are known. NodD-dependent NodO, a flavonoid-induced protein secreted by *R. leguminosarum* bv. viciae via a type I secretion system, was the first rhizobial protein whose role in symbiosis was elucidated (de Maagd 1989). NodO has been reported to confer host range extension in *Rhizobium* sp. (Vlassak et al. 1998).

In rhizobia, the type III secretion system is responsible for the secretion of nodulation outer proteins (NOPs). Besides flavonoids and NodD1, the *B. japonicum* type III secretion system also requires NodW (Krause et al. 2002). The functional type III secretion system has so far been reported in *S. fredii* USDA257 (Krishnan et al. 2003), *S. fredii* HH103 (deLyra et al. 2006), *Rhizobium* strain NGR234 (Viprey et al. 1998), *B. japonicum* USDA110 (Krause et al. 2002), *Bradyrhizobium elkanii* (Okazaki et al. 2009), and *Mesorhizobium loti* MAFF303999 (Okazaki et al. 2010). *S. fredii* USDA257 has been reported to secrete several NOPs including nopA, nopB, nopC, nopL, nopP, and nopX via the type III secretion system (Deakin and Broughton 2009). In *Rhizobium* sp. strain NGR234, a promiscuous symbiont capable of nodulating 112 genera of legumes (Pueppke and Broughton 1999) and removal of all secreted or specific NOPs significantly diminishes its nodulation ability on many of its hosts (Ausmees et al. 2004). Several studies have established that proteins secreted by the type III secretion system can have both positive and negative influence on the rhizobial nodulation ability of legume both at the genus and species level (Viprey et al. 1998; Krause et al. 2002; Krishnan et al. 2003; Okazaki et al. 2009, 2010).

Surface Polysaccharides Contribute to Various Stages of Symbiotic Development

Rhizobia produce four different surface polysaccharides – extracellular polysaccharides (EPS), lipopolysaccharides (LPS), K polysaccharides (K antigens, capsular polysaccharides, or KPS), and cyclic glucans. These polysaccharides play an important role at various stages of symbiotic development including the colonization of root, recognition of host, formation of infection thread, and invasion of nodule (Cooper 2007). EPS are essential during the early infection process of the symbiotic development, but at a later stage such as the penetration of the infection thread into the cortical cells or the setting up of the nitrogen-fixing phenotype, LPS are known to play a specific role. Flavonoids are not required for the synthesis of surface polysaccharides, but they act to influence the final structures and symbiotic activity of these compounds either during or after their biosynthesis (Cooper 2007). The role of surface polysaccharides in the establishment of the legume-rhizobia symbiosis has been comprehensively reviewed by Fraysee et al. (2003).

Rhizobial Secretome Also Contains Several Other Important Compounds

Rhizobia also produce several other compounds such as the flavonoid-, NodD1-, NodD2-, and SyrM2-dependent IAA in *Rhizobium* sp. NGR234 (Theunis et al. 2004); pentacyclic triterpenoid lipids, hopanoids, in bradyrhizobia and *Rhizobium* sp. (Kannenberg et al. 1995); a quorum sensor-AHL and a non-AHL quorum sensor, bradyoxetin, in bradyrhizobia (Loh et al. 2002; Mathesius et al. 2003); and a

plant growth enhancer, lumichrome, in *S. meliloti* (Phillips et al. 1999; Matiru and Dakora 2005). These compounds are required at different stages of the symbiotic development from root colonization to the formation of a functioning nodule (Cooper 2007).

Transporters Are Necessary for the Secretion of Root Exudates

Many compounds including flavonoids often have to be transported for exudation into the rhizosphere from their site of synthesis in the cytoplasm. Contrary to earlier opinion, secretion of phytochemicals into the rhizosphere is now believed to involve both primary and secondary transporters. Sugiyama et al. (2007), using a specific ABC transporter inhibitor, sodium orthovanadate, showed that secretion of the flavonoid genistein was dependent on ATP which suggests the involvement of ABC transporters in the secretion processes. This report was supported by the work of Badri et al. (2009) using *Arabidopsis* knockout mutants lacking expression of specific ABC transporter proteins. Their study not only provides direct evidence of the ABC transporters in the process of root secretion but also showed that structurally different compounds can be transported by one ABC transporter, or different transporters could transport one compound. ABC transporters comprise one of the largest protein families ubiquitously present in all phyla (Higgins 1992). The largest numbers of ABC transporters (more than 100) are encoded in the plant genome (Kang et al. 2011). Functional ABC transporters are generally made up of two hydrophobic transmembrane domains (TMD) and two cytosolic domains (Martinoia et al. 2002). Initially ABC transporters in plants were thought to be exclusive for detoxification processes (Martinoia et al. 1993), but it is now established that they are also involved in diverse processes as in the response to pathogen, deposition of surface lipid, accumulation of phytate in seeds, transport of flavonoids, and transport of the phytohormones auxin and abscisic acid (Sugiyama et al. 2007; Kang et al. 2011).

Root Exudates in Mycorrhizal Symbiosis

More than 80 % of all terrestrial plants are known to be associated with mycorrhizal fungi. Mycorrhizal associations are broadly divided into two groups – endomycorrhizal (arbuscular mycorrhiza, AM) and ectomycorrhizal associations. The association is mutually beneficial with the fungi helping the plant acquire nutrients such as phosphate from the soil which were otherwise unavailable to the plant and the plant in turn supplying photosynthates to the fungi. AM symbiosis, by far, is one of the most prevalent and the oldest known plant symbioses and is believed to have played an important role in the land colonization of plants. Here, we will be focusing on AM symbiosis due to their similarity with the legume-rhizobia symbiosis. Based on fossil records they are thought to have evolved 400–450 mya (million years ago) (Remy et al. 1994). Approximately 6,000 species of fungi and 200,000 plant species are known to be involved in mycorrhizal association (Wang and Qui 2006; Bonfante and Anca 2009). Hyphae produced by AM fungal soil propagules, asexual spores, or mycorrhizal roots initiate the AM symbiosis when they colonize compatible root.

The establishment of AM symbiosis also involves chemical communication facilitated by root exudates similar to the legume-rhizobia symbiosis. Owing to their presence in root exudates, flavonoids have been suggested to be involved in the signaling in AMF symbiosis. Various workers have reported the role of flavonoids in hyphal growth, hyphal differentiation, and root colonization (Morandi 1996 and Vierheilig et al. 1998). The stimulatory effect of flavonoids on hyphal growth is determined by the structure of flavonoids, and the presence of CO_2 enhances this effect (Becard et al. 1992; Chabot et al. 1992; Poulin et al. 1993; Scervino et al. 2006). Colonization of plant roots by mycorrhizal fungi results in the level of flavonoids (Vierheilig et al. 1998). Interestingly, there seems to exist some genus-species specificity among fungi to alter the pattern of flavonoids in roots

during the establishment of AM symbiosis (Morandi et al. 1984; Larose et al. 2002). Generally, the level of flavonoids is known to follow a predictable pattern where the concentrations of flavonoids are usually found to be highest at the later stage of AM symbiosis (Larose et al. 2002). However, the role of flavonoids as signal molecules in AM symbioses is now under a cloud with the recent identification of strigolactones as host recognition signals for AM fungi.

Strigolactones: Evolving Role in AM Symbiosis

In AM symbiosis, exchange of nutrients between the partners occurs through the arbuscules or the highly branched haustoria. The roots of the host plant release specific hyphal branching-promoting compounds, a prerequisite for root colonization in AM fungi (Giovannetti et al.1993; Harrison 2009). Reception of these compounds by the fungi results in the release of signal compounds both by the plant and the fungi called *branching factors* and *Myc factors*, respectively, required for successful colonization (Buee et al. 2000; Kosuta et al. 2003, Maillet et al. 2011) (Fig. 13.2). With the successful isolation and characterization of 5-deoxystrigol, Akiyama and Hayashi (2006) were the first to provide evidence of the probable involvement of strigolactones in promoting hyphal branching in AM fungi. The extensive work of Besserer et al. (2006, 2008, 2009) later corroborated this finding. The germination of spores was reported to be enhanced by the presence of strigolactones in *Glomus intraradices* and *Glomus claroideum.* Strigolactones have also been suggested to be involved in directing the growth of fungi towards roots (Akiyama and Hayashi 2006). Despite the probable existence of other alternative mechanisms, it is now established that the induction of fungal hyphal

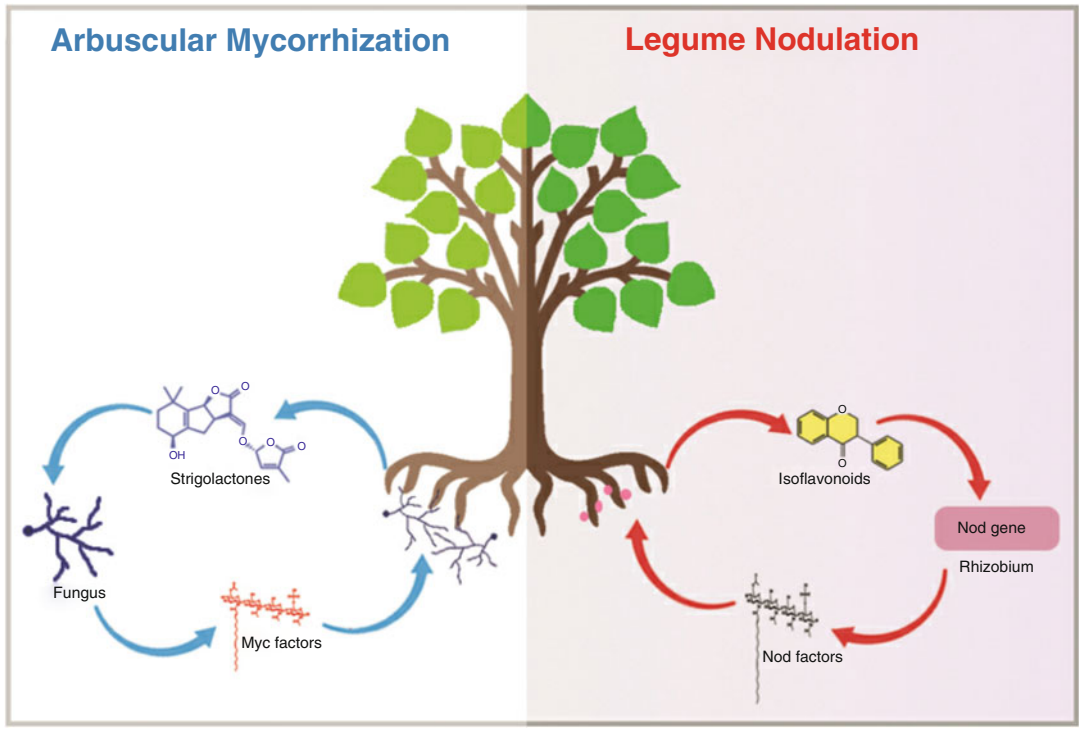

Fig. 13.2 Signaling pathway in the establishment of legume-rhizobia and AM symbioses. Specific isoflavonoid secreted by roots of legume induces the production of a lipo-chito-oligosaccharide nodulation signal (Nod factor) in rhizobia triggering a series of cascading events which ultimately lead to the development of a functional root nodule. Strigolactones are considered as the signal molecules from the host plant in the AM symbiosis

branching by strigolactones is an important step in AM symbiosis (Giovannetti et al. 1994; Gomez-Roldan et al. 2007). The acquisition of nutrients by plants was initially believed to be regulated by strigolactones (Gu et al. 2011; Koltai and Kapultnik 2011; Umehara 2011). However, the recent work of Yoneyama et al. (2012) with the strigolactone-deficient *ccd8* mutant indicates that strigolactones are not essential for nutrient regulation of mycorrhizal symbioses. Foo et al. (2013) also observed that suppression of mycorrhizal colonization could not be altered by strigolactone-deficient *ccd8* mutants and strigolactone response *rms4* mutants. The characterization of two fungal hyphal branching inducers 2-hydroxydodecanoic acid and 2-hydroxytetradecanoic acid in the root exudates of carrots suggests the presence of other branching factors besides strigolactones (Nagahashi and Douds 2011). Concentration as low as 10 nM was sufficient for the strigolactone analogue GR24 to induce nuclear division and enlargement of mitochondria and rapid increase of NADH content, NADH dehydrogenase activity, and ATP content in *Glomus rosea,* suggesting the presence of a sensitive strigolactone perception system in AM fungi (Besserer et al. 2006, 2008; Gutjahr and Parniske et al. 2013).

Conclusions

The plant rhizosphere teems with many microbes each affecting the other and in turn being influenced by plant derived factors. Legume-rhizobia and legume-mycorrhiza interactions are mutually beneficial associations in nature. These associations are facilitated by chemical cues exchanged between the host and the symbionts. In the rhizosphere, which includes plant roots and the surrounding area of soil influenced by the roots, plants exude chemicals to effectively communicate with their neighboring soil organisms. Some of these chemical cues are specific towards a particular organism, and others seem to be present commonly in root exudates of many plants. Rhizobia and mycorrhizae respond to such cues, the former specifically and the latter nonspecifically. However, there are many plant-microbe interactions taking place in the rhizosphere which have neither been studied nor understood well. The chemical nature of the root exudates seems to differentially regulate the microbial community and its activities in the soil. Research should now focus on characterizing and identifying chemical components in root exudates of various plants and especially legumes which have a stimulatory effect on other microbial interactions apart from rhizobia and mycorrhizae.

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Actinorhizal and Rhizobial-Legume Symbioses for Alleviation of Abiotic Stresses

14

Brahim Bouizgarne, K. Oufdou, and Y. Ouhdouch

Abstract

Plant symbiotic interactions with nitrogen-fixing bacteria could be used in agricultural and forest soils under harsh conditions and for land reclamation. These associations include rhizobia interacting with legumes species and the actinobacterium *Frankia* interacting with actinorhizal plants. These plants are distributed worldwide and suffer in many areas – especially in arid and semiarid regions from harsh biotic and abiotic conditions. Abiotic stress conditions include climatic conditions, soil characteristics, water and nutrient availability, salinity, and soil contamination by heavy metals, xenobiotics, and toxins. Thus, the improvement of symbiotic nitrogen fixation under stress conditions may rely on strategies devised to select more tolerant plant-bacteria associations and to use successfully efficient synergistic systems such as those involving mycorrhizal fungi and rhizobacteria, particularly plant growth-promoting rhizobacteria (PGPR). This chapter focuses on main stress conditions affecting plant-bacteria symbioses with emphasis on mechanisms that underpin plant resistance to stress. Biological strategies involving microbial inoculants aiming to protect and improve plant symbioses against detrimental effects of abiotic stresses so as to enhance the crop production in such conditions are discussed. In addition, plant-bacteria symbioses can also be used to promote the establishment of perennial vegetation that might limit the heavy metal pollution of soils and consequently represent an effective management procedure in disturbed soils. This chapter also focuses on main techniques successfully used for the purpose of land reclamation by plant-bacteria symbioses.

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Introduction

Most subtle known plant-bacteria associations are dinitrogen-fixing symbioses. They occur in nature as associations involving certain gram-negative bacteria (rhizobia) and legumes (legume-rhizobia symbioses) or between the gram-positive bacteria of the genus *Frankia* (order *Frankiales*, class *Actinobacteria*) and woody dicotyledonous actinorhizal plants (Pawlowski and Sirrenberg 2003). Diverse bacteria, commonly named rhizobia, are known to infect and nodulate legumes, while only genus *Frankia* is associated with actinorhizal plants as nodulating microorganism. Currently, 98 species of legume-nodulating bacteria belonging to 13 bacterial genera and 13,000 legume species from 700 genera are identified (Rajwar et al. 2013). Among these plants, soybean (*Glycine max*) is the dominant crop legume, representing 50 % of the global legume crop area. Taxonomically, rhizobia belong to the family *Rhizobiaceae* (α-subclass of *Proteobacteria*) and include species of the genera *Rhizobium*, *Bradyrhizobium*, *Ensifer* (formerly named *Sinorhizobium*), *Mesorhizobium*, *Allorhizobium*, and *Azorhizobium*. Currently, an ongoing number of rhizobia are being discovered. Recent findings indicate that rhizobial bacteria are diverse and also include *Devosia*, *Methylobacterium*, *Ochrobactrum*, *Phyllobacterium*, and *Shinella*. Some legumes are nodulated by strains of *Burkholderia*, *Cupriavidus*, and *Herbaspirillum* species belonging to the β-subclass of *Proteobacteria* (Chen et al. 2003; Masson-Boivin et al. 2009).

Actinorhizal plants comprise 200 plant species belonging to 25 genera of woody plants from eight non-leguminous dicotyledonous angiosperm families *Casuarinaceae*, *Betulaceae*, *Rhamnaceae*, *Rosaceae*, *Myricaceae*, *Coriariaceae*, *Elaeagnaceae*, and *Datiscaceae* (Chaia et al. 2010). While most known legume plants produce seeds and fodder with high protein content and exert beneficial effects on crop productivity in diverse cropping ecosystems over the world (Zahran 1999; Terpolilli et al. 2012), actinorhizal plants are of great ecological importance. They occupy ecological niches where legumes do not thrive and are mainly used for reclamation of degraded lands and as a source of fuel and wood. Also, *Frankia* species are considered as pioneer microorganisms in soils and as early successional species following deglaciation or fires (Chapin et al. 1994; Roy et al. 2007). Among actinorhizal plants, *Casuarinaceae* and *Betulaceae* families are the most widely planted around the world. *Casuarina* species, which are Mediterranean trees that can reach a final height of 20–30 m, are among the fastest growing trees in the world (can grow up to 2–3 m per year). *Casuarina equisetifolia* is the most widespread and the best known species. Members of this genus are used as soil binder in landslide areas and planted extensively in coastal areas of tropical and subtropical regions as windbreaks against wind erosion (Diem and Dommergues 1990). Wood from these plants is used for carpentry, construction, and fuel production. Some plants produce edible fruits, and leaves are used as fodder. For example, in India, 5,000,000 ha is planted with *C. equisetifolia* and produces 10 million tons of pulpwood (Karthikeyan et al. 2009).

Biological nitrogen fixation (BNF) by N_2-fixing plants is a sustainable and environmental-friendly alternative to chemical fertilization. By supplying combined nitrogen to plants, microbes forming association contribute to plant health and productivity in agricultural and forest soils. It is generally accepted that more than 60 % of the N input to plants has a biological origin and more than 30 % of the biological N input to plants is due to the activity of symbiotic plant-bacteria systems (Barea et al. 2005). Annual levels of at least 35 million tons of nitrogen are attributed to rhizobia-legume symbiosis (Freire 1992) corresponding to a 25–30 % of global annual terrestrial N inputs. On the other hand, it is estimated that actinorhizal plants are responsible for as much as 15–25 % of global nitrogen fixation (Dawson 2008). In most agricultural systems, the symbiotic interactions of legumes-rhizobia provide 80 % of the primary source of biologically fixed N (Sadowsky and Graham 1998). Regarding crop and forestry sustainment,

it is well known that many countries are using bacterial-plant symbioses as fertilizers in agriculture. Thus, many researches are aimed to select more efficient nitrogen-fixing plants, able to grow in agrosystems with low available nutrients. These biological strategies were encouraged by the major public concern for reducing agrochemical inputs as they constitute a threat to global environment. Indeed, these plants are nonpolluting and more cost-effective soil fertilizers compared to chemical fertilizers and sewage sludge. In addition, these associations could succeed in many areas where harsh abiotic conditions are inimical to the growth of many plants. Abiotic stress conditions in relatively harsh environments include harsh climatic conditions, soil characteristics, water and nutrient availability, salinity, and soil contamination by heavy metals, xenobiotics, and toxins. These symbioses deploy various resistance mechanisms to combat the abiotic stress conditions. These mechanisms underlie physiological and metabolic shifts that help plant to adapt to stress conditions. The most important responsive trait is the involvement of antioxidant enzymes such as peroxidase and catalase that have the ability to remove free radicals and help plant to mitigate oxidative damage and consequently to overcome stress conditions.

Plant bacterial symbioses are applied to alleviate abiotic stresses in many crop systems. It is well known that inoculation with indigenous N_2-fixing bacteria increases soil fertility and quality especially in poor soils and can contribute to the plant tolerance to abiotic environmental stresses (Richards et al. 2002; Mnasri et al. 2007; Bianco and Defez 2009; Tani and Sasakawa 2003; Oufdou et al. 2014). Efforts are made to search for new stress-tolerant crops or genetically engineered plants. However, as yet these strategies are costly and out of reach for developing countries. Easier and cheaper strategies are based on the application of microbial inoculants. Thus, efforts are made to select more tolerant rhizobia or *Frankia* strains and/or effective plant associations. For efficient applications of this strategy, it is suggested that bacterial strain–plant genotype combination should be considered for selecting the most adapted microbe-plant combinations to environmental stress conditions (Requena et al. 1997; Valdenegro et al. 2001; Remans et al. 2008). Apart from this, other soil microbiota are also able to interact with N_2-fixing plants. They involve plant growth-promoting bacteria (PGPB) and mycorrhiza (Barea and Azcón-Aguilar 1983; Probanza et al. 1996; Yamanaka et al. 2003; Han and Lee 2005; Aroca et al. 2007; Saravanakumar and Samiyappan 2007; Solans 2007; Egamberdiyeva and Islam 2008; Figueiredo et al. 2008; He and Critchley 2008; Marulanda et al. 2009). The simultaneous occurrence of these microorganisms together with the N_2-fixing bacteria generates a multipartite relationship able to significantly improve nitrogen fixation and the host plant growth. Bioaugmentation by stress-tolerant rhizobacteria particularly PGPR could be used as a stress alleviation mechanism. One interesting mechanism of action of PGPR is mediated by 1-aminocyclopropane-1-carboxylate (ACC) deaminase, an enzyme that cleaves ACC, the precursor molecule of ethylene. By this mechanism, the level of ethylene in a stressed plant is lowered. In addition, rhizobacterial species have the ability to elevate the levels of stress enzymes in the plant. Such mechanism and others confer to plant a tolerance commonly named "induced systemic tolerance" (IST) (Yang et al. 2009). Associations with mycorrhizae are commonly called tripartite associations. Dual inoculation of these microorganisms with rhizobia or *Frankia* strains resulted often in tolerance compared to those formed by the bacterial symbiont alone. Indeed, PGPR and mycorrhizae play important roles in the life of plants, not only ensuring more efficient nutrition but also improving plant health through increased protection against biotic and abiotic stresses. Increasing extension of salinized and contaminated/degraded lands is of global concern. N_2-fixing plants and their associations are also used for mitigating negative impact of toxicity in heavy metal-contaminated soils and for soil bioremediation.

There are many comparative features of the legume-*Rhizobium* symbiosis with those of the actinorhizal symbiosis regarding physiological resistance to stress conditions and their ability to interact with microbial inoculants able to enhance their tolerance (N_2-fixing symbiont bacteria and beneficial soil microbiota). Thus, this chapter focuses on main resistance mechanisms deployed by these two main plant-bacteria symbioses and their applications or their associations with beneficial telluric microorganisms (mainly PGPRs and mycorrhizae) under soil stressed systems. This chapter also focuses on main applications of plant-bacteria symbioses in contaminated soils and advances in soil bioremediations. Figure 14.1 summarizes mechanisms deployed by N_2-fixing bacteria, PGPRs, and AM fungi to alleviate abiotic stresses.

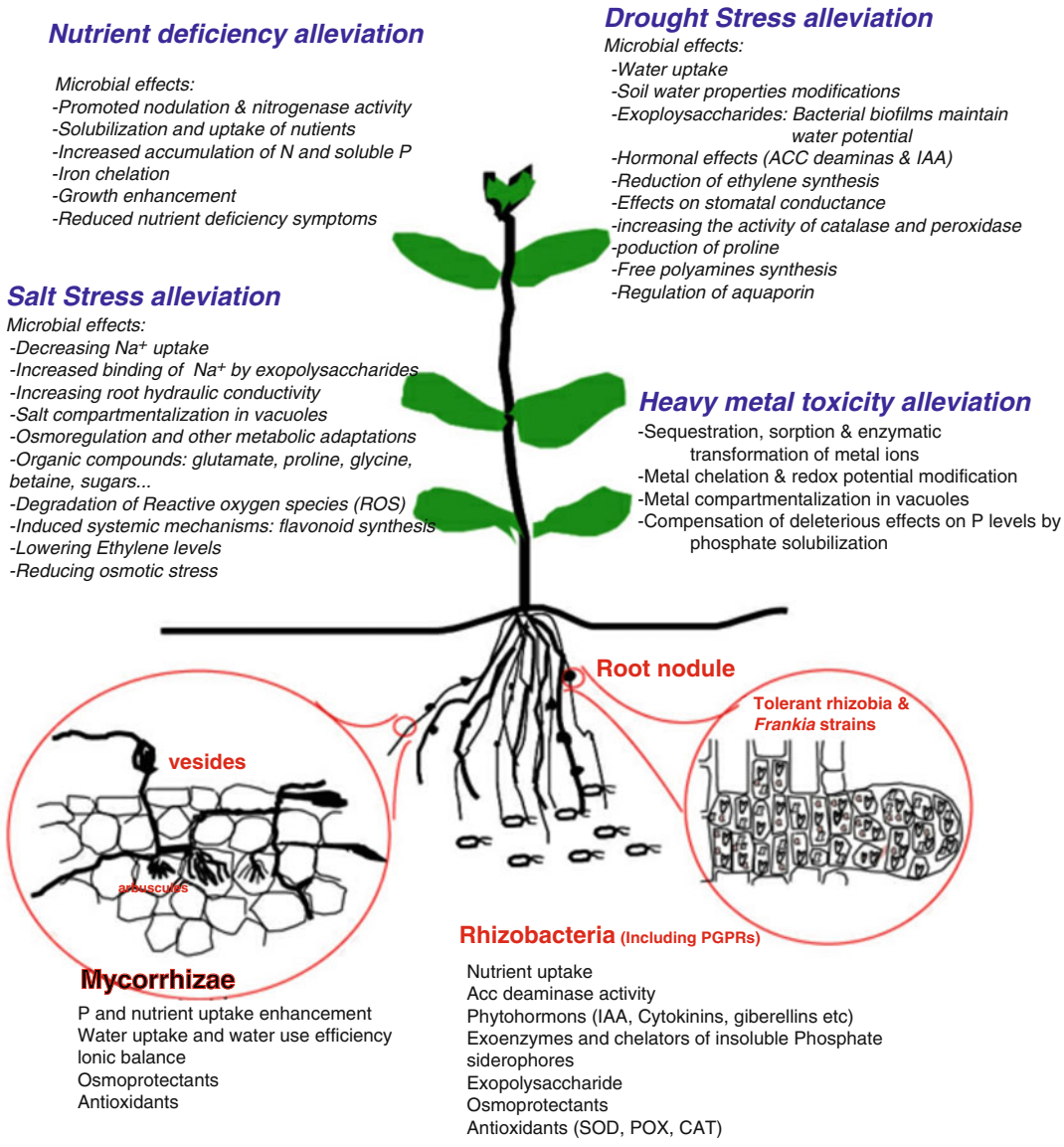

Fig. 14.1 Scheme summarizing the mechanisms deployed by N_2-fixing bacteria, PGPRs, and AM fungi to alleviate abiotic stresses. *CAT* catalase, *IAA* indoleacetic acid, *PGPR* plant growth-promoting rhizobacteria. *POX* peroxidase, *SOD* superoxide dismutase

Application of Plant-Bacteria Symbioses to Alleviate Abiotic Stresses

Tolerance and Growth Promotion Under Nutrient Deficiency

Soil nitrogen status is considered as one of the main factors affecting sustainability of plant growth in a crop or forest ecosystem. N status of soils under N-fixing plants including actinorhizals and legumes is often considered as high. They are very important in agriculture and forestry as they are responsible for a substantial part of the global flux of nitrogen from atmospheric N_2 to fixed mineral forms. Alders are, for example, of major interest for soil nitrogen status as their leaf litter is highly N rich and therefore mineralized much faster than that of other tree species (Claessens et al. 2010). However, in many nutrient-poor areas, colonization of primary successional substrates is largely limited by low levels of nutrients. In most soils where low levels of available nitrogen are a major limitation, N_2-fixing plants which are the primary source of fixed nitrogen (Zahran 1999; Barritt and Facelli 2001) could succeed. Nitrogen fixation and root nodule formation are substantially affected by soil nutrient availability of mineral N (Arnone et al. 1994; Bollman and Vessey 2006) and P (Reddell et al. 1986a; Sanginga et al. 1989; Yang 1995; Kouas et al. 2005; Sadowsky 2005). It is well known that nodulation is stimulated in poor N soils, whereas high levels of mineral forms of nitrogen (nitrate and ammonium) have a negative effect on nodulation and nitrogen fixation activity (Arnone et al. 1994; Baker and Parsons 1997; Wall et al. 2000; Tiffany and Williams 2005; Bollman and Vessey 2006; Shamseldin and Moawad 2010). Nodule development requires P consumption for DNA and plasma membrane synthesis, for cell division, and also for symbiotic N_2 fixation due to the need for large amount of ATP for nitrogenase activity (reduction of N_2). In general, the fixation of atmospheric N_2 in plant-microbe symbioses can negatively be impacted by phosphorus limitation (Reddell et al. 1986a; Tang et al. 2001). Indeed, it was demonstrated that nodulation and growth in some actinorhizal plants are frequently limited by P in early successional environments (Chapin et al. 1991; Uliassi et al. 2000). Also, *Frankia* infective abilities following deglaciation were only showed if P was supplemented (Chapin et al. 1994). Phosphorus has been shown to have a positive effect on nodulation in many actinorhizal and legume plants (Wall et al. 2000; Olivera et al. 2004; Gentili et al. 2006). This was also observed in some soils from Australia, where Reddell et al. (1986a) found that nodulation occurred only when P amendments were performed.

N_2-fixing plants able to grow under nitrogen deficiency are considered as important for revegetation in such soils as these can accumulate nitrogen. Different perennial legumes were studied for their potential use in revegetating Australian soils with phosphorus deficiency (Pang et al. 2010). In addition, efforts have been made to select efficient legume plants for N_2 fixation and biomass and seed yield, particularly under conditions of N and P deficiency (Elisondo Barron et al. 1999; Tang et al. 2004; Remans et al. 2008). The selection of N_2-fixing plants tolerant to N deficiency in combination with tolerant bacterial symbionts seems to be a valuable strategy. Consequently, screening and inoculation of efficient N_2-fixing bacteria in order to provide improvement in plant production equal to or better than that of nitrogen fertilization are aspects that have been investigated by numerous researchers (Sellstedt and Huss-Danell 1986; Romdhane et al. 2008).

Many researches are aimed to select and apply PGPRs for their beneficial effects on plant fitness and productivity. These rhizobacteria are well known to promote plant growth by various direct and indirect mechanisms including phytohormone synthesis and phosphate solubilization and are consequently used as phytostimulators and biofertilizers for agricultural crops. In addition, the use of microbial inoculants could represent an alternative strategy to the use of chemical fertilizers notably NP-based fertilizers. Rhizobia can also be considered as PGPR endophytes as they contribute to plant growth. However,

researchers prefer to denote PGPR as free root-colonizing rhizobacteria. Most known PGPRs belong to the genera *Arthrobacter*, *Bacillus*, *Micromonospora*, *Pseudomonas*, and *Streptomyces*. Some of these bacteria were applied for growth stimulation and nodulation in both legume and actinorhizal plants (Solans 2007; Malik and Sindhu 2011). The most important traits of PGPRs in association with N_2-fixing plants are the improvement of nodulation (Knowlton and Dawson 1983; Zhang et al. 1996; Lucas-Garcia et al. 2004) and supply of soluble P by solubilization (Toro et al. 1998; Valverde et al. 2006). The application of PGPRs to N_2-fixing plants often resulted in enhanced legume or actinorhizal plants' growth. Hence, fitness was enhanced in various crops when PGPRs were co-inoculated with rhizobia or with *Frankia* (Knowlton and Dawson 1983; Probanza et al. 1996, 1997; Solans 2007; Wani et al. 2007a; Dardanelli et al. 2008; Egamberdieva et al. 2010; Malik and Sindhu 2011). An example of such application is the single, dual, or triple seed inoculations of common bean (*Phaseolus vulgaris*) with *Rhizobium leguminosarum* bv. *phaseoli*, N_2-fixing *Bacillus subtilis*, and P-solubilizing *Bacillus megaterium* in a highland region in Turkey as indicated by significantly increased nodulation, plant growth, nutrient uptake, and seed yield equal to or higher than nitrogen (N), phosphorus (P), and NP mineral fertilizer application. Moreover, triple inoculations were most efficient for enhancement of macro- and micronutrient uptake resulting in an increase in mineral concentration in the plant and seeds (Elkoca et al. 2010). Similar results were found in *Vicia faba* L. inoculated with four different *Rhizobium*/-*Azospirillum* and *Rhizobium*/*Azotobacter* combinations which led to changes in mineral macro- and micronutrients, K, P, Ca, Mg, Fe, B, Mn, Zn, and Cu, compared with plants inoculated with *Rhizobium* alone (Rodelas et al. 1999). Recently, Solans et al. (2011) reported that three root-associated saprophytic actinomycetes (*Streptomyces* sp., *Actinoplanes* sp., and *Micromonospora* sp.) stimulated nodulation by *Frankia* in the actinorhizal plant *Ochetophila trinervis*. These root associated actinobacteria produced phytohormones (indole acetic acid (IAA), gibberellic acid, and zeatin) at levels higher than produced by the symbiotic *Frankia* strain alone. One interesting strain *Micromonospora* MM18 showed direct promotion effect on shoot growth of *Ochetophila trinervis* when inoculated as mycelium as well as cell-free growth medium (Solans 2007). However, in some cases, effects of dual inoculation with *Rhizobium* and PGPR depend on various parameters including legume species, PGPR species, and the nature and concentration of secondary metabolites released by PGPR. Co-inoculation with *Pseudomonas fluorescens* 2137 increased colonization of *Bradyrhizobium japonicum* on soybean roots. Nodule numbers and acetylene reduction increases were observed probably due to release of growth-promoting substances by *Pseudomonas* that stimulate *B. japonicum*, whereas co-inoculation with another strain *P. fluorescens* WCS365 had an opposite effect (Chebotar et al. 2001). Under field conditions, co-inoculation of PGPR, *Serratia* and *P. fluorescens* with *R. leguminosarum*, had positive effects on lentil but had no effect on pea (Chanway et al. 1989). These opposite effects are likely due to PGPR species and/or legume plant species. Elo et al. (2000) isolated a number of bacteria from the humus of spruce and tested their potential beneficial effects on alder (*Alnus incana*), red fescue (*Festuca rubra*), and birch (*Betula pendula*).

Under stress conditions of soil nutrient deficiency, the effectiveness of PGPRs as bioinoculants for N_2-fixing plants has been demonstrated in legume plants. Positive effects were reported in relation to iron, phosphorus, magnesium, calcium, and the nutrient uptake under iron-limiting conditions. Siderophores produced by certain bacteria could contribute to enhanced availability of iron. Inoculation of mung bean (*Vigna radiata*), with the siderophore-producing *Pseudomonas* strain GRP3, resulted in a reduced chlorotic symptoms and enhanced chlorophyll level (Sharma et al. 2003). In general, differences in the responsiveness to PGPR among cultivars of legumes were observed. Thus, PGPRs could be used efficiently for enhancement of tolerance to P deficiency in non-tolerant cultivars. In the

P. vulgaris genotype DOR364, a poor nitrogen fixer under P deficiency, Remans et al. (2008) succeeded to partly alleviate this constrain by using a combination of *Rhizobium etli* and *Azospirillum* inoculation. However, in another *Phaseolus* genotype, the beneficial effect of co-inoculated *R. etli* and *Azospirillum* was shown to be dependent on P nutrition (Remans et al. 2007). These authors showed that co-inoculation resulted in efficient growth enhancement only at high P conditions and combinations failed under low P conditions. It is also worth mentioning that in general, the interplay between *Rhizobium*, PGPR species, and plant host species plays a major role in the success of these combinations under P stress conditions. Under deficient P conditions, while combination of *Rhizobium* and *Azospirillum* was ineffective, the beneficial effect of co-inoculation of *Rhizobium* with *Pseudomonas putida* was strongly expressed (Remans et al. 2007).

In addition, it is well known that N_2-fixing plants can also harbor ecto- or endomycorrhizae forming either tripartite or tetrapartite associations. These kinds of associations were found in both legume plants and actinorhizae (Vance 2001; Yamanaka et al. 2003; Roy et al. 2007; He and Critchley 2008; Gherbi et al. 2008), and their importance for plant growth promotion in poor soils has been documented extensively. Their beneficial effects on plant growth are mainly due to the positive effects of the mycorrhizal fungi through P supply (Barea et al. 2005). In many cases, dual inoculation of actinorhizal plants with arbuscular mycorrhizal fungi (AMF) **a**nd *Frankia* resulted in a more enhanced growth than single inoculation with *Frankia* alone (Vasanthakrishna et al. 1994; Oliveira et al. 2005; Elumalai and Raaman 2009; Muthukumar and Udaiyan 2010; Orfanoudakis et al. 2010). In addition, the usefulness of AMF co-inoculation with N_2-fixing bacteria is well established in nutrient-poor soils. Inoculation of mycorrhiza fungi promotes the growth of actinorhizal plants by various mechanisms including increased uptake of phosphorus (Theodorou and Reddell 1991; He and Critchley 2008; Elumalai and Raaman 2009). It was demonstrated that *Elaeagnus* and *Shepherdia* inoculated with soil containing *Frankia* and mycorrhizal fungi under nutrient deficiency had a superior nodulation than those uninoculated (Visser et al. 1991). Theodorou and Reddell (1991) found that symbiotic mycorrhizae could contribute to a successful *Frankia* symbiosis in *Casuarina* plants by increasing N_2 fixation especially in P-deficient soils, in arid climates. However, inoculation experiments showed that mutual interactions and delays between inoculations of the two symbionts could play a major role in the efficiency of tripartite association (Gardner 1986; Theodorou and Reddell 1991). Theodorou and Reddell (1991) observed that *Amanita* sp. formed ectomycorrhizae on *Allocasuarina littoralis* and *Casuarina cunninghamiana* but not on *Casuarina equisetifolia*. It was also shown that the intensity of ectomycorrhizal infection with *Pisolithus tinctorius* and *Laccaria laccata* was higher in *C. equisetifolia* rather than *C. cunninghamiana* suggesting specificity between the plant and mycorrhizae. In addition, under limited supply of mineral N and P, Sempavalan et al. (1995) found a high degree of coordination between *Frankia* and *Glomus* for nodulation and mycorrhizal colonization of roots of *C. equisetifolia*, while Gardner (1986) reported a possible competition between *Frankia* and AMF microsymbionts for infection sites and/or nutrients in AMF-colonized *C. equisetifolia* resulting in negative effect on nodulation. It was thus suggested that efficiency of these tripartite associations in poor soils could be ensured if the moments of inoculations of both symbionts are skillfully monitored (Gardner 1986). All these observations emphasize the importance of knowledge of specific combinations involving mycorrhizae/*Frankia*/actinorhizal plant species.

Alleviation of Drought Stress

Arid or semiarid land areas are increasing worldwide due to climate change and global warming. This condition has deleterious effects on plants in these areas. Drought stress affects plant hormone balance by decreasing the endogenous cytokinin level and increasing the levels of abscisic acid

(ABA) content in the leaves. This effect leads to stomatal closure. Many N_2-fixing species undergo deleterious physiological effects of water deficiency and show a reduction in nitrogen fixation when subjected to drought (Zahran 1999; Sharma et al. 2010). In addition to its deleterious effects on nodules and nitrogenase activity (Sundström and Huss-Danell 1987; Devries et al. 1989; Porcel et al. 2003), drought could affect the survival of rhizobia and the size of the soil population of infective *Frankia* and their infectivity (Mahler and Wollum 1981; Sayed et al. 1997). Drought also alters the soil properties and renders it unsuitable for soil microbial activity and crop growth (Selvakumar et al. 2012).

Actinorhizae are more drought tolerant than other nitrogen-fixing microorganisms. Numerous actinorhizal plants mainly those belonging to the genus *Casuarina* were reported as drought tolerant (Diem and Dommergues 1990). Though they are high water demanders, alders (*Alnus*) are well adapted to water deficits due to their root systems (Claessens et al. 2010). The performance of tolerant *Casuarina* under conditions of drought could be due to their high rate of photosynthesis and decreased stomatal conductance. Thus, researches focused on propagation of *Casuarina* species tolerant to drought via in vitro tissue culture for seedling production or via cuttings of plant parts.

Numerous legume plants are adapted to drought stress conditions. Among these are arid legumes, such as cluster bean (*Cyamopsis tetragonoloba*), dew bean (*Vigna aconitifolia*), and cowpea (*Vigna unguiculata*) (Kumar 2005). It was observed that numerous drought-tolerant species (cultivars) are characterized by a deeper root profiles, a greater root biomass, and a greater root-to-shoot ratio. Those variabilities could be exploited in breeding programs for N_2 fixation drought tolerance. In addition, efforts are made to select more tolerant N_2-fixing plant species to drought by using conventional selection and breeding (El-Lakany 1983; Pimentel et al. 1999; Sinclair et al. 2001; Khan et al. 2010; Asfaw et al. 2012), and efforts are also made to screen more tolerant *Frankia* and rhizobia strains (Sayed et al. 1997; Romdhane et al. 2009; Zahran 2001; Sayed 2011). For instance, salt-tolerant rhizobia that were able to nodulate chickpea (*Cicer arietinum*) were selected for their efficiency in conditions of water deficiency (Romdhane et al. 2009). Mnasri et al. (2007) reported that when inoculated to *P. vulgaris*, a salt-tolerant *Ensifer meliloti* induced more tolerance to drought than a salt-sensitive bacterial strain *Rhizobium tropici*. *Frankia* may be absent in some arid soils, whereas non-leguminous N_2-fixing plants are often nodulated in well-hydrated areas. Thus, there is a need to inoculate host plants with a selected microsymbiont in arid and semiarid areas. Attempts to select more tolerant *Frankia* strains were performed by Sayed et al. (1997) who found that the infectivity of a soil inoculum decreased by storage under dry conditions and pointed out the importance of screening *Frankia* strains for drought tolerance during preparation of inoculum for use in hot climates. Thereafter, inoculation of tolerant bacterial symbionts was also used as a strategy to alleviate drought stress in many N_2-fixing plants. Bacterial aggregation as biofilms in the vicinity of roots was reported as a mechanism that could contribute to maintain convenient water potential (Khan et al. 2011). Bacteria producing extracellular materials were reported for their beneficial effects. Exopolysaccharides (EPS) act as adhering material that help bacteria to attach to soil particles and by binding soil particles together lead to soil aggregate formation. EPS contribute to hold water within soil aggregate and thus lead to ameliorate plant growth under drought conditions. Among EPS-producing bacteria, rhizobia were applied by Hartel and Alexander (1986). They observed a significant correlation between the levels of EPS produced by cowpea *Bradyrhizobium* strains and their drought tolerance.

On the other hand, rhizobacteria and mycorrhizae were also investigated for their ability to help plants to alleviate drought stress. PGPR bacteria could also contribute to drought alleviation in legume plants through hormonal effects that involve IAA and ACC deaminase. Marulanda et al. (2009) used three bacteria: *Pseudomonas sp*., *P. putida*, and *B. megaterium* with IAA-producing abilities to alleviate drought stress in

Trifolium repens. Alleviation effect could also involve the regulation of ethylene synthesis by ACC-deaminase-producing *Pseudomonas* which showed to be effective in both pot and field trials (Arshad et al. 2008). Indeed, reduction of ethylene synthesis by such bioinoculants effect could result in better growth under drought stress (Zahir et al. 2008). ACC-deaminase-producing rhizobacteria could obviously reduce the negative effects of ethylene on nodulation and N_2 fixation. Dual inoculation involving PGPRs and rhizobia proved their effectiveness in mitigating water stress deficiency in N_2-fixing plants. In bean, Figueiredo et al. (2008) used dual inoculation of *Paenibacillus polymyxa* and *R. tropici* to alleviate drought stress. Plants co-inoculated with *Rhizobium* and *Paenibacillus* exhibited greater nodulation and nitrogen content compared to drought-stressed plants inoculated with only *Rhizobium*. In addition, PGPR induced systemic mechanisms to drought could represent a powerful tool to alleviate such alterations. IST by increasing the activity of catalase and peroxidase in water stress was found in *P. fluorescens* Pf1-treated green gram plants (*V. radiata* L.) (Saravanakumar et al. 2012).

Mycorrhizae could also be used as bioinoculants for drought stress alleviation (Ruiz-Lozano et al. 2001). Several studies have stressed their role in improving legume and actinorhizal plant tolerance to drought (Goicoechea et al. 1998; Ruiz-Lozano et al. 2001; Valdenegro et al. 2001; Zhang et al. 2010). Mycorrhizal inoculation significantly improved both nodulation and nitrogen fixation in these conditions (Azcón et al. 1988; Theodorou and Reddell 1991; Ruiz-Lozano et al. 2001). In soybean, mycorrhizae were reported to have antioxidant activities and to protect legume plants against premature nodule senescence induced by drought stress (Porcel et al. 2003). It was also established that they could reduce the production of proline, a marker of the water stress effect in plants (Goicoechea et al. 1998). In addition, they induce free polyamine synthesis (Goicoechea et al. 1998). This contributes to increased accumulation of nonstructural carbohydrates that could serve as osmoprotectants by lowering the osmotic potential under drought conditions. The regulation of root hydraulic properties by AM symbiosis under drought stress is also correlated with the regulation of aquaporins which are membrane intrinsic proteins that form a pore in cell membranes of living organisms (Aroca et al. 2007). Uehlein et al. (2007) found an AM-induced gene expression of specific aquaporin genes in mycorrhized *Medicago truncatula*. The application of mycorrhizae for drought tolerance in actinorhizal plants was also reported (Zhang et al. 2010). Zhang et al. (2010) found that AM associations improve the drought tolerance of *C. equisetifolia* seedlings by lowering the permeability of plasma membrane and malondialdehyde contents, enhancing concentrations of P nutrition, soluble sugars, soluble proteins, and peroxidase activities. However, efficient dual inoculation involving symbiotic mycorrhizae and rhizobia seems to be variable according to bacterial interactions in a given combination. Ruiz-Lozano et al. (2001) reported that dual inoculation of *Bradyrhizobium* and *Glomus intraradices* was less effective than dual inoculation with *G. mosseae*, in alleviating drought-induced nodule senescence in soybean plants, suggesting the existence of a competitive interaction between the very aggressive *G. intraradices* and *Bradyrhizobium*. The importance of efficient combinations was also discussed by Valdenegro et al. (2001) who used combinations of three mycorrhizae and one PGPR, *Enterobacter* sp., and found that the positive effects of AMF isolates on growth of *Medicago arborea* plants under drought conditions were stimulated by the co-inoculation. This effect was however found to be dependent on the inoculated rhizobial strain and on the AMF isolate suggesting the importance of efficient combinations of AMF, PGPR, and rhizobia in such conditions. Table 14.1 presents some selected examples of drought stress alleviations in legume plants by rhizobia and microbial inoculants (i.e., rhizobacteria or mycorrhizae).

Alleviation of Salt Stress

Salinity in soil is one of the main environmental stress parameters, especially in arid and semiarid

Table 14.1 Selected examples of drought stress alleviation in legume plants by rhizobia and microbial inoculants

Stress	Plant species	Symbiotic bacteria	Microbial inoculant	References
Drought	*Glycine max* (soybean)	*Bradyrhizobium japonicum*	*Glomus mosseae*	Ruiz-Lozano et al. (2001)
	Medicago arborea	*Rhizobium meliloti*	Arbuscular mycorrhizal fungi and *Enterobacter* sp.	Valdenegro et al. (2001)
	Medicago sativa L. cv. Arag6n (Alfalfa)	*R. meliloti*	*Glomus fasciculatum*	Goicoechea et al. (1998)
	Phaseolus vulgaris (common bean)	*Rhizobium tropici*	*Paenibacillus polymyxa*	Figueiredo et al. (2008)
	Vicia faba (faba bean)	*R. leguminosarum* bv. *viciae*	*Azotobacter chroococcum*	Dashadi et al. (2011)

regions, and can severely reduce crop production. Salinization is mainly due to human activities such as excessive tillage or irrigation with saline water. Over 800 million hectares of land throughout the world is salt affected (Rengasamy 2006) with over 70 million ha being farmlands (FAO 2005). The osmotic stress is currently one of the most serious environmental factors in agriculture (Manchanda and Garg 2008). In legume plants, salt toxicity results in reduction in survival and productivity (Shannon 1998; Yuan et al. 2007), whereas actinorhizae, particularly *Casuarina* species, are well known for their relatively high resistance to salt stress conditions (Luard and El-Lakany 1984; Graves and Gallagher 2003; Diagne et al. 2013). Many reports stressed that toxicity is mainly due to plant uptake of salts. Under elevated salt levels, movement of water in response to the water potential gradient results in a water stress. It was stressed that salinity above 50 mM NaCl has deleterious effects on legume plants. It was also reported that nodule formation in N_2-fixing plants is very sensitive to salt stress (Zahran and Sprent 1986; Young et al. 1992). Salinity also affects nitrogenase activity in legumes (Cordovilla et al. 1999) and actinorhizal plants (Sande and Young 1992; Oshone et al. 2013) and consequently reduces N_2 fixation. For instance, in the common bean, high Na^+ and Cl^- concentrations were found in nodules and leaves (Ashraf and Bashir 2003). Nodule formation is even more sensitive than the rhizobia themselves (Zahran 1991). Moreover, in the nodule formation process, it appears that the ability of the rhizobia to infect roots and nodule initiation are more sensitive to salt stress than nodule development (Zahran and Sprent 1986; Craig et al. 1991). Consequently, salinity affects plant performance resulting in reduction in shoot and root growth and legume crop production (Oufdou et al. 2014). In addition, salinity could aggravate this effect through increase in levels of ethylene, a stress hormone that significantly reduces plant growth. Salinity can also indirectly exert deleterious effects by restricting the carbon supply from the host plant as a result of photosynthesis inhibition (Soussi et al. 1999). Other deleterious effects are calcium deficiency, production of reactive oxygen species (ROS), tissue necrosis, and increase in ABA levels that lead to early senescence of older leaves.

Several works emphasized that salt tolerance depends on both plant and bacterial species. For example, most reports agreed that fast-growing rhizobial strains are more tolerant than slow-growing strains (Ghittoni and Bueno 1996). Members of the fast-growing genus *Rhizobium* are generally more tolerant to salts than members of the genus *Bradyrhizobium*. It is also well known that some legumes, e.g., *Sesbania aculeata*, *V. faba*, and *G. max*, are more salt tolerant than other legume species such as *Pisum sativum*. In a given species, tolerance could be variable according to cultivars (Soussi et al. 1999). Resistance to saline conditions is the result of adaptive processes and is mainly due to ability to exclude Na and Cl ions and to prevent the transport of these ions to shoots. They also involve control of ion transport and compartmentation and osmotic solute synthesis and accumulation, which lead to osmotic adjustment. Development of plant symbiont-tolerant systems for cultivation in salt-affected soils is currently considered as

necessity. Indeed, several studies are aimed at identifying salt-tolerant genotypes belonging to legumes (Moreno et al. 2000; Sadiki and Rabih 2001) and actinorhizal plants (El-Lakany and Luard 1982; Luard and El-Lakany 1984; Nambiar-Veetil et al. 2011). It is thus well established that N_2-fixing plant symbiosis tolerance to salt stress conditions is variable among species or cultivars. Moreno et al. (2000) found a large genotypic variability in bean cultivars for salt tolerance at the seedling stage and identified some salt-tolerant cultivars with higher performances. Fast-growing salt-tolerant actinorhizal plants showing variable tolerance levels could also be used in salt-affected terrains. Actinorhizal trees such as alders (Graves and Gallagher 2003) and members of the *Casuarinaceae* (*C. equisetifolia* and *C. glauca*) are able to grow under saline conditions (Nambiar-Veetil et al. 2011), whereas *Myrica cerifera* are intolerant to high salinity (Young et al. 1992). Tani and Sasakawa (2006) found that in *C. equisetifolia* plants, which are highly tolerant to salt stress, proline is primarily synthesized as a major compatible solute to adjust the osmotic pressure when Na^+ accumulates in the cells, allowing to maintain cell homeostasis under salt stress conditions. Currently, several members of the *Casuarinaceae* are used in the reclamation of salt-affected lands, and more recent researches focused on tissue culture of salt-tolerant *C. equisetifolia* clones (Liu et al. 2003) and creation of transgenic trees that are more tolerant to salt in tropical regions (Nambiar-Veetil et al. 2011).

Greater performance of N_2-fixing plants under saline conditions seems to be determined mainly by the tolerance of the host plant and its interaction with the bacterial symbionts (Zahran 1991; Tani and Sasakawa 2003). Consequently, inoculation with more tolerant symbiotic bacterial strains with efficient nodulation and N_2 fixation under saline conditions may contribute to the compensation of the salinity-induced plant growth loss (Girgis et al. 1992; Hafeez et al. 1999; Zahran 2001; Tani and Sasakawa 2003). Therefore, the selection of osmotolerant strains of symbiotic bacteria able to efficiently nodulate plants under saline conditions is considered as a great challenge to improve the productivity of N_2-fixing plants. Several salt-tolerant *Rhizobium* and *Frankia* species have been reportedly isolated that could tolerate saline environments (Rai 1983; Hafeez et al. 1999; Zahran 1999; Tani and Sasakawa 2003; Srivastava et al. 2012; Oshone et al. 2013). Among them, some strains of *Rhizobium meliloti* and *R. fredii* (Kassem et al. 1985) and *Sinorhizobium* (*Ensifer*) *arboris* (isolated from *Acacia*) (Zahran et al. 1994) were able to grow at NaCl concentration of more than 300 mM. Similarly, Hafeez et al. (1999) described a *Frankia* strain CcO1 that could tolerate up to 500 mM NaCl (Ec_e of 47 dSm-1), and Tani and Sasakawa (2003) reported nodulation of *C. equisetifolia* with a selected strain of *Frankia* Ceq1 under saline conditions up to 300 mM (28 dSm^{-1}). Accumulation of various solutes was correlated to tolerance of *Rhizobium* (Zahran 1999). Among accumulated compatible solutes are K^+, glutamate, proline, glycine betaine, proline betaine, trehalose, and the dipeptide N-acetylglutaminylglutamine amide. Recently, the role of poly-β-hydroxyl butyrate (PHB) in cell protection of rhizobia during high saline conditions was reported (Arora et al. 2006). Glycine betaine was found to increase more in the salt-tolerant strains of *R. meliloti* than in sensitive strains (Smith et al. 1988). It has also been suggested that rhizobia synthesizing IAA may prevent the deleterious effects of salinity. Bianco and Defez (2009) reported an increased tolerance against salt stress by *M. truncatula* nodulated by a strain of an IAA-overproducing mutant of *Sinorhizobium meliloti*. This effect was accompanied by higher amount of proline and accumulation of antioxidant enzymes in mutant inoculated plants in comparison with plants inoculated with the wild strain. Such inoculation with symbiotic bacteria could help plants to tolerate salt conditions. For instance, in arid saline soils, it was reported that *B. japanicum* S2492 has significantly increased dry weight, plant height, and yield (>35 %) of soybeans (Egamberdiyeva et al. 2004).

The application of other beneficial microbes as inoculants have recently gained interest as beneficial microbes could help plants to mitigate

Table 14.2 Selected examples of salinity stress alleviations in legume plants by combinations of rhizobia and rhizobacteria

Salinity	*Galega officinalis* (goat's rue)	*Rhizobium galegae* sv. *officinalis*	*P. extremorientalis* or *P. trivialis*	Egamberdieva et al. (2013a)
	P. vulgaris (common bean)	*R. tropici* or *R. etli*	*Azospirillum brasilense*	Dardanelli et al. (2008)
	P. vulgaris (common bean)	*R. tropici*	*Chryseobacterium balustinum*	Estevez et al. (2009)
	P. vulgaris (common bean)	*Ensifer fredii*	*C. balustinum*	Estevez et al. (2009)
	Glycine max (soybean)	*B. japonicum*	*Pseudomonas putida*	Egamberdieva et al. (2013b)
	Cicer arietinum (chickpea)	*Rhizobium*	*Azospirillum brasilense*	Hamaoui et al. (2001)

stress conditions. Hamaoui et al. (2001) reported that inoculation with the PGPR *Azospirillum brasilense* significantly enhanced nodulation by native rhizobia in chickpea. This bacterial treatment was shown to significantly enhance growth parameters and to reduce the negative effects on plant growth caused by irrigation with saline water. Dual inoculation including rhizobia and bacterial inoculants could also be used to alleviate salinity stress conditions in N_2-fixing plants (Dardanelli et al. 2008; Estevez et al. 2009; Egamberdieva et al. 2013b). In soybean, Estevez et al. (2009) reported that co-inoculation of the PGPR *Chryseobacterium balustinum* and *Ensifer fredii* led to a significant increase in the number of nodules and root growth under moderate saline conditions. This symbiotic performance was more pronounced than in single inoculation with *E. fredii*. Similar results were found by Egamberdieva et al. (2013b) in soybean co-inoculated by *B. japonicum* and *P. putida*. PGPRs include bacteria with ACC-deaminase activity (Shaharoona et al. 2006; Saravanakumar and Samiyappan 2007; Egamberdieva et al. 2013a). Saravanakumar and Samiyappan (2007) reported that *P. fluorescens* with ACC-deaminase activity led to plant growth promotion and enhanced saline resistance in groundnut plants compared with that inoculated with *Pseudomonas* strains lacking ACC-deaminase activity. PGPR effects could also be mediated by "induced systemic mechanisms" to salt. Indeed, through mechanisms such as osmoregulation and other metabolic adaptations, PGPR could ameliorate the tolerance of plants to saline conditions (Egamberdiyeva and Islam 2008). Phenolic compounds are well known as resistance mechanisms, and their elicitation by PGPRs was reported in many research works as accompanying salinity alleviations in co-inoculated plants (Dardanelli et al. 2008, 2012). Enhanced synthesis of flavonoids (known as phenolic substances involved in *Rhizobium* attraction), following PGPR inoculation, was reported by Dardanelli et al. (2008). They showed that co-inoculation of *A. brasilense* and *R. tropici* strain CIAT899 or *R. etli* ISP42 resulted in enhanced nitrogen fixation concomitant to production of a variety of flavonoid species in *P. vulgaris* cv. *Negro jamapa* under salt stress. This effect is linked to an efficient activation of nod factor genes under such conditions. It was also reported that most of PGPRs could produce EPS able to bind Na^+. Han and Lee (2005) observed that inoculation of EPS-producing strain resulted in a decrease in Na^+ content of soybean grown under saline conditions. The production of osmoprotectants, a kind of organic compounds under inhibitory osmolarities, by either rhizobia or beneficial bacteria that are subsequently taken up by plants, is also reported by several authors. Table 14.2 presents selected examples of salinity stress alleviations in legume plants by combinations of rhizobia and rhizobacteria.

Tripartite association involving ectomycorrhizal and endomycorrhizal fungi was also reported as a powerful tool to alleviate saline stress. Colonization by AMF can help plants to cope

with salinity stresses (Ruiz-Lozano 2003) by compensating for its negative effects on nodulation and N_2 fixation (Ruiz-Lozano and Azcón 1993). It was reported for instance that dual inoculation with AM and rhizobia decreased the deleterious effects of sulfate salinity on plant growth and on P and N accumulation in *Lathyrus sativus* (Jin et al. 2010). Zhong et al. (2010) demonstrated that the ectomycorrhizal fungus (EMF) *P. tinctorius* increased *Casuarina* tolerance to salt condition. One mechanism of osmotic stress tolerance induced by AMF colonization was shown by Aroca et al. (2007) in bean (*P. vulgaris*) for which an increase in root hydraulic conductivity of the host plants was observed. Reddell et al. (1986b) observed that dual inoculation of *Frankia* and the EMF *Suillus* excludes salt and toxic metals from metabolic pathways by compartmentalization in vacuoles and cell walls.

Alleviation of Heavy Metal Toxicity in Contaminated Soils

Several soil systems are contaminated by mineral compounds such as heavy metals or by organic compounds of various origins. Many of these compounds originate from industrial and intensive agriculture activities and discharges of untreated or partially treated wastewater. It is well known that heavy metals even in traces are toxic against organisms (Giller et al. 2009; Nonnoi et al. 2012) including humans and result in major health problems. The most common heavy metal contaminants are cadmium (Cd), chromium (Cr), cupper (Cu), mercury (Hg), lead (Pb), nickel (Ni), manganese (Mn), and zinc (Zn). The elevated levels of heavy metals in the environment are excessively absorbed by roots and translocated to shoots, leading to impaired metabolism and reduction in plant growth (Rodríguez-Llorente et al. 2010; Perez-Palacios et al. 2013). For instance, it was reported that in legume plants, Cd contamination could result in a decreased uptake of nutrients, inhibition of various enzyme activities, and induction of oxidative stress (Sandalio et al. 2001) or inhibition of chlorophyll synthesis and photosynthesis (Padmaja et al. 1990). For actinorhizal plants, inhibition of growth, nodulation, and nitrogenase activity by heavy metals such as Cd (Hensley and Carpenter 1987), Cu (Fessenden and Sutherland 1979), Ni (Wheeler et al. 2001), and Zn (Cusato et al. 2007) have been reported. It is currently accepted that the sensitivity to a given heavy metal is variable according to plant species. In *Alnus crispa*, Cu concentration of 60 mg kg^{-1} caused a reduction of nodule dry weight, while nodulation was totally inhibited at 100–150 mg kg^{-1}. However, Lorenc-Pluciñska et al. (2013) described two species of *Alnus*, *A. incana* and *A. glutinosa* tolerant to Cu concentrations higher than 1,500 mg kg^{-1} of soil. Unfortunately, heavy metals cannot be destroyed biologically. The physical-chemical methods to remediate heavy metal pollution are not suitable for practical applications. In addition to their high costs, they led to a large destruction of soil structure and fertility and soil properties. Thus, biological remediation presents the advantage to be of low-cost, effective, eco-friendly, and socially accepted strategy.

Nitrogen-fixing plants could play a key role in the detoxification of heavy metals (Lorenc-Pluciñska et al. 2013; Vivas et al. 2003a, b). Among strategies that should be adopted prior to introduction of N_2-fixing plants in contaminated sites is the selection of tolerant plant species and/or bacterial symbionts. Actinorhizal plants such as alders have been used for the reclamation of sites contaminated with toxic levels of trace elements (Lefrancois et al. 2010; Babu et al. 2013; Lorenc-Pluciñska et al. 2013). Lorenc-Pluciñska et al. (2013) reported that two actinorhizal species *A. incana* and *A. glutinosa* grow well in heavily polluted soil. In spite of accumulation of toxic metals in their roots and nodules and a decreased rate of N_2 fixation, concentrations of Cu, Zn, Cd, and Pb in leaves of both alder species were within normal ranges. In fact, heavy metals are accumulated in roots of these species, and only small proportions are transferred to aboveground parts, including leaves. These species of alders can be recommended for the revegetation of areas polluted with phytotoxic concentrations of trace metals. However, these results were obtained only from short-term pot trials and

Table 14.3 Selected examples of heavy metal bioremediation by legume and actinorhizal plants in association with microbial inoculants

Plant	Symbiont bacterium	Tolerant microbial inoculant species	Heavy metal contamination[a]	Reference
Alnus glutinosa (alder)	Soil as *Frankia* inoculum		Cu	Whitbread-Abrutat (1997)
A. glutinosa (alder)	*Frankia* (Ni tolerant)		Ni	Wheeler et al. (2001)
A. glutinosa and *Alnus incana* (tolerant plants)	*Frankia*		Cu and Pb	Lorenc-Pluciñska et al. (2013)
Alnus firma (alder)		*Bacillus thuringiensis* (endophyte)	Pb, Zn, As, Cd, Cu, and Ni	Babu et al. (2013)
Pisum sativum (pea)	*Rhizobium* (metal tolerant)		Ni, Zn	Wani et al. (2008)
Trifolium (clover)	*Rhizobium*	PGPRs and the mycorrhizal fungus *Glomus mosseae*	Zn	Vivas et al. (2006a)
Lupinus luteus (lupine)	*Bradyrhizobium*	*Pseudomonas* and *Ochrobactrum cytisi*	Cd, Cu, and Pb	Dary et al. (2010)
Pisum sativum (pea)		*Pseudomonas brassicacearum* and *P. marginalis*	Cd	Safronova et al. (2006)
*Trifolium pratens*e (red clover)	*Rhizobium*	*Brevibacillus* sp.	Pb	Vivas et al. (2003a)
Trifolium repens (white clover)	*Rhizobium*	*G. mosseae* (mycorrhizae) and *Brevibacillus* sp.	Cd	Vivas et al. (2003b)
T. repens (white clover)	*Rhizobium trifolii*	*Brevibacillus brevis* and *Glomus mosseae* (AM fungus)	Ni	Vivas et al. (2006b)
Vigna radiata (mung bean)		*P. putida*	Cd, Pb	Tripathi et al. (2005)
V. radiata (mung bean)	*Bradyrhizobium* (metal tolerant)		Ni, Zn	Wani et al. (2007b)

[a]*Cd* cadmium, *Cu* copper, *Mn* manganese, *Ni* nickel, *Pb* lead, *Zn* zinc

require further field validation. *Frankia* and rhizobia strains were also consequently screened for their tolerance to heavy metals (Richards et al. 2002; Carrasco et al. 2005; Dary et al. 2010; Bélanger et al. 2011). Heavy metal-tolerant *Frankia* could allow actinorhizal plants to colonize contaminated terrains, and their spectra of tolerance were reported to vary depending on strains tested (Richards et al. 2002; Bélanger et al. 2011). Richards et al. (2002) investigated the tolerance of 12 *Frankia* strains to a wide variety of heavy metals. While all of the 12 strains were sensitive to low concentrations (<0.5 mM) of Ag^{+}, AsO_2^{-}, Cd_2^{+}, SbO_2^{-}, and Ni_2^{+}, most were less sensitive to Pb^{2+} (6–8 mM), CrO_4^{2-} (1.0–1.75 mM), AsO_4^{3-} (>50 mM), and SeO_2^{2-} (1.5–3.5 mM). These authors also found that four strains are resistant to elevated levels of Cu^{2+} (up to 20 mM for strain CN_3). Carrasco et al. (2005) and Dary et al. (2010) have isolated effective rhizobia resistant to heavy metals from polluted soils in Spain. These strains could be exploited for revegetation programs of many other polluted areas.

Other microbial inoculants such as PGPRs or mycorrhizae could act as helper microbia in heavy metal-contaminated areas. Table 14.3 presents selected examples of heavy metal bioremediation by legume and actinorhizal plants in

combination with microbial inoculants. PGPRs as bioinoculants proved their utilities to alleviate heavy metal stress conditions and were used as a tool for rhizoremediation in contaminated soils. For instance, elevated levels of heavy metals interfere with P uptake by plants. By using P-solubilizing PGPRs, this effect could be compensated (Gupta et al. 2001). Rhizobacteria have been shown to possess several characteristics that can alter heavy metal bioavailability through acidification of the microenvironment, releasing of chelating substances, and modifying heavy metal redox potential (Abou-Shanab et al. 2003). One of the major traits of these bacteria is the sequestration and accumulation of heavy metals (Richards et al. 2002; Gupta et al. 2004; Lee et al. 2006). It was suggested to be the role of polysaccharides or proteins as potential binding sites for these heavy metals (Richards et al. 2002). Works by Vivas et al. (2003a, b, 2006a) showed the utility of bioinoculants for alleviation of heavy metals as a strategy to grow legumes in contaminated soils. In these studies, bacteria with the ability to survive and colonize the rhizosphere were used in Zn-, Pb-, or Cd-contaminated soils. Clover (*Trifolium*) growing in soil contaminated with Cd inoculated with a Cd-adapted autochthonous PGPR, *Brevibacillus*, resulted in growth-promoting effects and a reduction in Cd transfer from soil to plants (Vivas et al. 2003b). A significant enhancement of nitrogen and phosphorus accumulations and nodule formation concomitant to decrease the amount of Pb absorbed by plants was also reported (Vivas et al. 2003a). Dary et al. (2010) used *Lupinus luteus* and a consortium of metal-resistant PGPR co-inoculated (including *Bradyrhizobium*, *Pseudomonas* sp., and *Ochrobactrum cytisi*) for reclamation of multi-metal-contaminated soil. They observed root accumulation of heavy metals concomitant with increment in lupine growth promotion effect when compared to *Lupinus* inoculated with *Bradyrhizobium* alone. This mixture also succeeded to reduce plant toxicity symptoms and metal accumulation in both shoots and roots. The alleviating effect could also be due to more intimate bacteria-plant relationships such as those ensured by endophytic PGPRs. Babu et al. (2013) reported a significant enhancement of heavy metal phytoremediation by *Alnus firma* with an endophytic strain of *Bacillus thuringiensis*.

Tripartite associations involving mycorrhizae were also used as a tool to alleviate heavy metal stress and for bioremediation (Díaz et al. 1996; Roy et al. 2007; Karimi et al. 2011). Mycorrhizae can help contribute to phytoremediation by their ability to sequester heavy metals through the production of chelates or by absorption. Consequently, less heavy metals are translocated to plant shoots and leaves. AMF were used in association with *C. equisetifolia* to rehabilitate heavy metal-polluted soils (Karimi et al. 2011). Tripartite association established by *Alnus*, mycorrhizae-*Frankia*, was reviewed by Roy et al. (2007). In their work aiming to provide *Trifolium pretense* growth promotion and lead toxicity reduction by *Brevibacillus*, Vivas et al. (2003a) reported that association of bacteria and mycorrhizae could interact synergistically resulting in significant positive effects. They reported that mycorrhization of AM fungi was positively stimulated by the PGPR bacterium resulting in significant growth under Pb contamination. All these associations could provide a basis for selecting nodulating bacteria associated with other microbial inoculants that may have applications in formulating appropriate inocula, effective when used for phytoremediation and revegetation of contaminated soils, but also could be a tenable strategy for improving growth while avoiding high input of chemical fertilizers in such sites.

Conclusions

Enhancing application and management of biologically fixed N could result in very important environmental and economic benefits for the humanity. N_2-fixing plants have an exceptional ability to form symbiotic association with rhizobia and *Frankia*. Hence, they have been used on a broad scale so as to improve the nitrogen status of soils. Symbiosis could represent a key factor in abiotic stress conditions, and many researchers stressed the importance of both host specificity and environmental variations in determining

patterns of symbiont efficiencies under such conditions. Therefore, the selection of more tolerant plant/symbionts has been one of the multiple approaches. A valuable selection approach could be performed by targeting multitolerant rhizobia. Recently, Elboutahiri et al. (2010) isolated rhizobia from drought and salt-affected regions of Morocco that were tolerant to salinity, water stress, high temperature, acidity, and heavy metals. However, bacterial strain–plant genotype combination should be considered for selecting the most adapted microbe-plant combinations to stress conditions. On the other hand, the application of microbial inoculants to N_2-fixing plants is one of the most interesting eco-friendly strategies for agriculture and forestry sustainability. Plant inoculation with more tolerant strains of these bacteria could contribute to their tolerance to stress conditions (Figueiredo et al. 2008). In comparison with legume plants, the use and exploitation of the beneficial effects of PGPRs to actinorhizal plants is still a rarely used approach by researchers (Knowlton and Dawson 1983; Probanza et al. 1996; Rojas et al. 2002). Very little information is available on the effectiveness of PGPRs as bioinoculants for actinorhizal plants under stress conditions. This topic needs to be further investigated. Unexplored fluorescent *Pseudomonas* and other PGPR species with beneficial properties (e.g., phytohormone or siderophore production) (Arshad et al. 2008; Figueiredo et al. 2008) could contribute to more tolerance to abiotic stress conditions. There is in fact a need to explore more number of effective combinations of PGPR and *Frankia*. Also, the application of helper bacteria or PGPRs in association with actinorhizal plants for bioremediation of eroded or degraded areas following industrial waste pollution is another aim to be focused on.

In addition, tripartite associations involving mycorrhizae seem to be promising for sustainable agriculture and forestry based on N_2-fixing plants. To help actinorhizal plants to withstand abiotic stresses, mostly used bioinoculants in tripartite associations are mycorrhizae. In researches focusing on tripartite associations, one of the most important technical traits is the selection of the appropriate microbial inoculants (mycorrhizae and PGPRs). In addition, the use of compatible multiple microbial consortia including bacterial symbionts and fungal symbionts acting synergistically, providing various beneficial effects, is also a powerful strategic tool (Requena et al. 1997; Muthukumar and Udaiyan 2010). In general, the use of tripartite and tetrapartite associations is largely studied and applied in both legumes and actinorhizal plants. However, the use of these associations should be approached cautiously in relation to efficient combinations, particularly those involving mutual interactions between the N_2-fixing bacterium and the bioinoculant (rhizobacterium or mycorrhizae) (Requena et al. 1997; Gardner 1986; Ruiz-Lozano et al. 2001; Valdenegro et al. 2001; Remans et al. 2007).

In addition to classical rhizobia and *Frankia* strains, another way is to study and evaluate other endophytic bacteria potentially found in nodules (nodule inhabitants) in experiments aiming to alleviate abiotic stress effects. Recently, an actinobacterium, *Micromonospora*, was found in actinorhizal and legume nodules as holding nitrogenase activity and nifH-like gene sequences demonstrating its potential role in N_2 fixation (Valdès et al. 2005; Trujillo et al. 2006, 2010; Carro et al. 2012). To our knowledge, most works dealing with abiotic stress tolerance are aimed to select only known symbiotic bacteria, i.e., rhizobia and *Frankia*, and very little work has addressed the role of other nodule-colonizing bacteria. Special attention should be drawn on isolating these bacteria, particularly those tolerant to stress conditions. Their use as bioinoculants in combination with rhizobia or *Frankia* strains could help to emerge a new dimension into the application of microbial inoculants to N_2-fixing plants under abiotic stress conditions.

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Azospirillum: A Biofertilizer for Every Crop

15

Samina Mehnaz

Abstract

Azospirillum is known for its nitrogen-fixing and phytohormone production ability. It is one of very well-studied plant growth-promoting rhizobacteria, at lab scale to field. None of its species or strain is reported as human or plant pathogen. It is considered as safest bacteria which can be used as a biofertilizer at commercial level for several crops, especially cereals or grasses including wheat and rice which are of economic importance for the whole world. Some of its species are reported for phosphate-solubilizing ability and high salt tolerance. Fifteen of its species have been isolated from variety of hosts and environmental sources; however, a majority have been reported from plants. There are several reviews available on this organism; in this chapter, an overview of this organism covering its plant growth-promoting abilities, used as inoculum in lab and field experiments and used as a commercial biofertilizer for different crops, is provided.

Characteristic Features of Genus *Azospirillum* and Its Species

Azospirillum is a gram-negative curve rod, microaerophilic, non-fermentative, and a chemoorganotroph that belongs to class *Alphaproteobacteria*. This bacterium was first time isolated, reported, and named in 1978 (Tarrand et al. 1978). As the name reflects, it was described as nitrogen-fixing, curved rod-shaped organism. Two species, *A. lipoferum* and *A. brasilense*, were designated at that time. Since then, 15 species of this genus have been described. For a long time, only five species were known, but during the last 10–12 years, ten new species have been described. *Azospirillum* is known to be an organism which has been isolated from most of the parts of the world, mainly from rhizosphere and roots of the members of the family Gramineae including rice, wheat, corn, and sugarcane as most common hosts. Coffee, fruits, and orchids have also been reported as host of this bacterium but less frequently as compared to grasses. Two new species *A. picis* and *A. thiophilum* had been isolated from very different environment, i.e.,

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road tar and sulfide spring (Lin et al. 2009; Lavrinenko et al. 2010).

Azospirillum was first time described as a nitrogen fixer – ability to convert atmospheric nitrogen into ammonia. Later on, the second most common characteristic feature was phytohormone production, specifically auxins. Among 15 species, *A. palatum* is an exception, lacking the ability to fix nitrogen, as reduction of acetylene (method for the detection of nitrogen fixation) was not detected by it (Zhou et al. 2009). The production of indoles is not reported for *A. picis* and *A. palatum.* The difference in carbon source utilization is helpful for distinguishing among species. Most common carbon sources are sugars and sugar alcohols. Reis et al. (2011) provided a very comprehensive detail on their carbon source utilization.

During the last 3 years, genomes of *A. lipoferum* 4B (www.genoscope.cns.fr), *A. brasilense* CBG497, *A. brasilense* Sp245, *A. amazonense* Y2, and *Azospirillum* sp. B510 have been sequenced and published (Kaneko et al. 2010; Sant'Anna et al. 2011; Wisniewski-Dye et al. 2012). These sequences provided the insight into genetics of this genus. This information can be used not only to improve the strains but also to explore their potential for purposes other than agriculture, e.g., bioremediation. A comprehensive review about genetic and biochemical traits of *Azospirillum* has been published by Steenhoudt and Vanderleyden (2000). Recently, Reis et al. (2011) discussed about the genetic makeup of *Azospirillum*, mostly based on genome of *Azospirillum* sp. B510. In addition to *nif* gene cluster, *nod* gene has also been identified in *Azospirillum* spp. Genes for indole acetic acid (IAA) production and those involved in chemotaxis signal transduction are also reported. According to information based on *Azospirillum* sp. B510 genome, this genus has nine sets of *rrns* genes which is atypical for the members of α-proteobacteria. Quorum sensing is a very rare feature for this genus. Boyer et al. (2008) reported the N-acyl-L-homoserine lactone (AHL) production in two strains of *A. lipoferum*.

If we look at the published material about this genus, most of the information is based on two species, i.e., *A. brasilense* and *A. lipoferum*. It

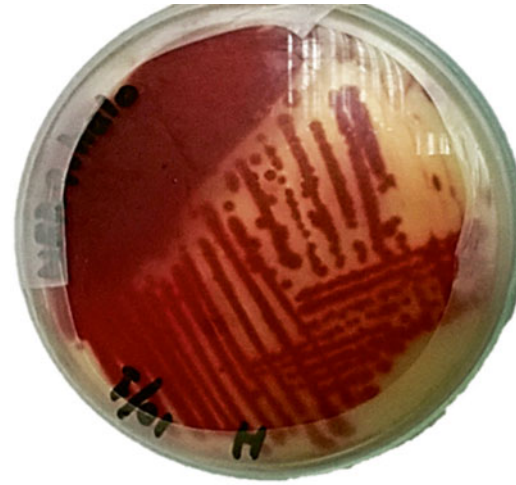

Fig. 15.1 Pure culture of *Azospirillum halopraeferens* NFP9 on LB medium (Mehnaz et al. unpublished results)

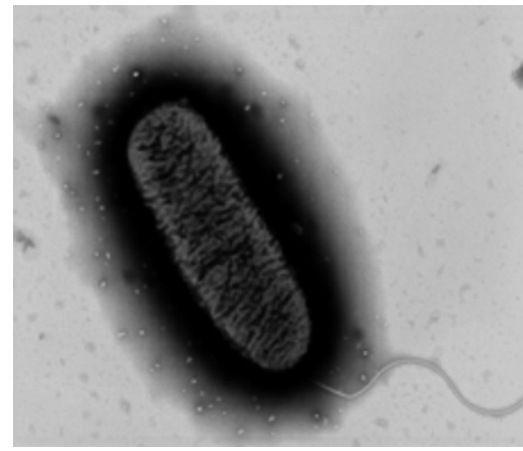

Fig. 15.2 Single cell of *Azospirillum zeae* N7 (Mehnaz et al. 2007a)

can be due to worldwide occurrence of these two as compared to the other species. *A. irakense* has not been reported from anywhere after its first isolation. Reports for *A. amazonense* and *A. halopraeferans* are also rare (recently authors' lab has isolated a strain of *A. halopraeferans* from *Kochia indica*; Fig. 15.1, unpublished results). Two of the species, *A. canadense* and *A. zeae*, have been isolated and designated by our group (Mehnaz et al. 2007a, b; Fig. 15.2) and not reported yet from anywhere else. Similarly, the rest of the species have been recently designated;

therefore, commenting on their occurrence is too early to discuss.

Growth-Promoting Abilities of *Azospirillum*

Among plant growth-promoting rhizobacteria (PGPR), *Azospirillum* is one of those bacteria which had never been reported as a pathogen for any living organism. It has been used as an inoculum for several crops due to its beneficial effects. These beneficial effects can be an individual or a combined effect of several mechanisms which exist in this bacterium. *Azospirillum* is known for nitrogen fixation, phytohormone production, phosphate solubilization, and siderophore production. It was never considered as a biocontrol agent although there were always reports available about its anti-pathogenic activity. ACC deaminase activity has not been reported for this genus, and quorum sensing is very rare as mentioned earlier. The growth-promoting attributes of *Azospirillum* are described below in detail.

Nitrogen Fixation

The most important characteristic feature of *Azospirillum* is its ability of biological nitrogen fixation (BNF). Nitrogen fixation is common among bacteria responsible for symbiotic relationship with leguminous plants. These bacteria are known for their endophytic nature and nodule formation and inability to fix nitrogen "in vitro." In contrast, *Azospirillum* is known as a "free-living" nitrogen fixer that does not make nodules. It stays in close association with the plant, in the rhizosphere, and on the root surface.

The most common method to detect the nitrogen fixation in bacteria is acetylene reduction assay. Nitrogenase enzyme responsible for nitrogen fixation has also the ability to convert the ethylene into acetylene. Acetylene reduction assay is based on it. N^{15} dilution method has also been used by several researchers and considered very authentic when it comes to quantifying the contribution of these microorganisms to the plants in terms of nitrogen and minimizing the use of N fertilizers. There is no doubt about the nitrogen-fixing ability of *Azospirillum*, but how much of the fixed nitrogen is transferred to the plants is still a big question. Transfer process is very slow, and a very small part becomes available to the plant. The death and subsequent mineralization of dead nitrogen fixers can release significant amount of fixed nitrogen (Okumura et al. 2013).

Phytohormone Production

Almost 80 % of soil bacteria have the ability to produce phytohormones, and *Azospirillum* is one of them. Phytohormones are divided into five classes, i.e., auxins, gibberellins, cytokinins, abscisic acid, and ethylene. *Azospirillum* is known for the production of all of these except ethylene.

Auxins

Auxins are responsible for division, extension, and differentiation of plant cells and tissues. These compounds increase the rate of xylem and root formation; control vegetative growth, tropism, and florescence; and affect photosynthesis, pigment formation, biosynthesis of several metabolites, and resistance to biotic stress (Bashan and de-Bashan 2010). Among auxins, IAA production is very high in this genus, and it follows more than one pathway.

IAA production by using tryptophan is known since 1979, for *Azospirillum* (Reynders and Vlassak 1979). IAA production depends on composition of medium, pH, and availability of tryptophan as precursor (Ona et al. 2003; Malhotra and Srivastava 2006, 2008). Spaepen et al. (2007) characterized IAA production via indole-3-acetamide (IAM) and indole-3-pyruvate (IPyA) intermediates, in *Azospirillum*. Very low level of pyridoxine and nicotinic acid increased IAA production in *A. brasilense*, indicating that vitamins may also regulate IAA synthesis (Zakharova et al. 2000). IAA is produced during all growth stages of *Azospirillum* and most after the stationary phase (Malhotra and Srivastava 2009). Due to this feature, credit of growth promotion by

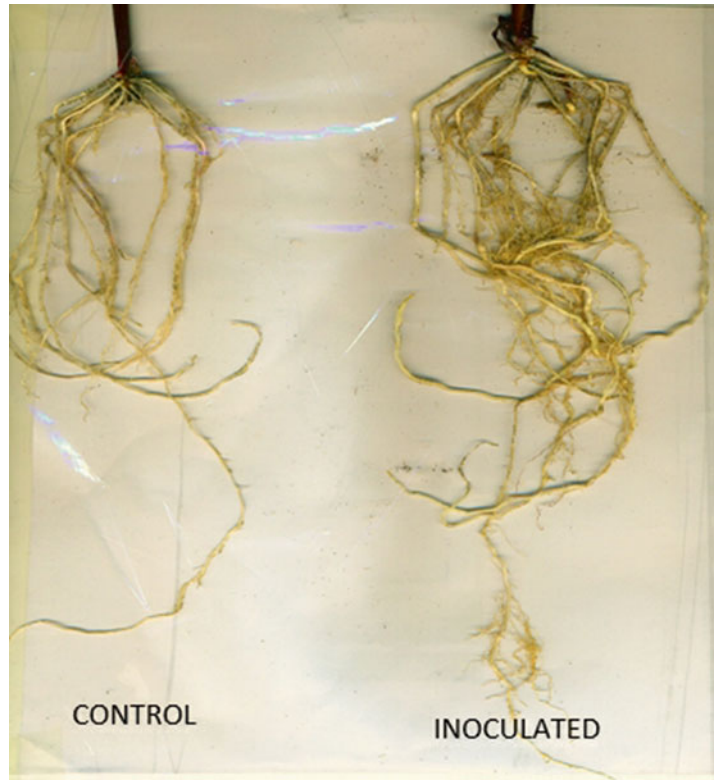

Fig. 15.3 Effect of *Azospirillum* sp. inoculation on root growth of corn plants (Mehnaz et al. unpublished results)

Azospirillum inoculation is given to IAA as effect lasts longer. Several auxins including indole butyric acid, indole lactic acid, indole acetamide, indole acetaldehyde, indole ethanol, indole methanol, and phenyl acetic acid (PAA) have been detected in *Azospirillum* cultures (Hartmann et al. 1983; Crozier et al. 1988; Fallik et al. 1989; Costacurta et al. 1994; Somers et al. 2005).

Plants inoculated with *Azospirillum* strains showed increase in number and length of root hairs, number of lateral roots, diameter and length of lateral and adventitious roots, and root surface area. IAA produced by *Azospirillum* is considered as the major mechanism responsible for increase in root system (Fig. 15.3; Mehnaz et al., unpublished results). Another possibility is that inoculation enhances the endogenous level of IAA in plant. The best way to prove the direct involvement of IAA produced by *Azospirillum* on plant growth is to develop IAA-deficient mutant and use them as inoculant, but due to existence of more than one pathway, it is almost impossible to make a mutant which does not produce IAA (Zakharova et al. 1999; Spaepen et al. 2007).

Gibberellic Acids (GAs)

GAs are responsible for cell division, cell elongation, and effects on different growth stages of plants. GAs are involved in breaking dormancy during seed germination. These compounds induce synthesis of α-amylase which hydrolyzes starch into glucose. *A. brasilense* is known to enhance germination of soybean and wheat seeds (Bacilio et al. 2003; Cassan et al. 2009a). It is reported that improved seed germination coincides with high GA production in *A. brasilense* cultures (Cassan et al. 2009b).

Azospirillum synthesizes and metabolizes GAs in vitro and in planta. The production of different GA compounds and metabolism of exogenously applied GA are reported for different *Azospirillum* species (Reis et al. 2011). Unlike IAA production, the mechanism of GA production is not known. However, dwarfism induced in maize plant by inhibitors of GA biosynthesis was

reverted by inoculation with *A. lipoferum* and *A. brasilense* (Lucangeli and Bottini 1997). Recently, Manivannan and Tholkappian (2013) isolated *Azospirillum* strains from tomato rhizosphere and estimated GA production from 3.3 to 9.5 µg/25 ml broth. GA_3 is the main GA identified in *Azospirillum*. Piccoli et al. (1999) reported the effect of water potential on GA_3 production. Water potential and oxygen concentration affect GA_3 production in *A. lipoferum;* production is reduced at high water potential and low O_2 concentration. Lucangeli and Bottini (1997) suggested the involvement of GA_3 produced by *Azospirillum* in growth promotion of maize.

Cytokinins

Cytokinins are derived from N-substituted amino-purines. In plants, these are produced in root tips and germinating seeds. These are transported in shoots to regulate cell division, shoot and root morphogenesis, chloroplast maturation, cell enlargement, leaf expansion, auxiliary bud release, and delaying senescence (Spaepen et al. 2009). Auxin is known to regulate the biosynthesis of cytokinin, and ratio of auxin to cytokinin is crucial during cell division and differentiation of plant tissues. Cytokinin production in defined culture medium has been reported for *Azospirillum* by several researchers (Tien et al. 1979; Horemans et al. 1986; Cacciari et al. 1989; Strzelczyk et al. 1994). Inoculation effect of these strains on plant is based on the balance between both the hormones produced by both the partners (Reis et al. 2011). Some *Bradyrhizobium* strains use cytokinins for nodulation (Giraud et al. 2007). *Azospirillum* being a cytokinin producer might support nodulation when co-inoculated with rhizobia as also reported earlier (Yahalom et al. 1990).

Abscisic Acid (ABA)

The main role of ABA is as a phytohormone produced in response to environmental stress, e.g., decreased soil water potential, heat, and water or salt stress. ABA produced in roots is translocated to leaves and alters osmotic potential of stomata guard cells leading to closing of stomata, preventing further water loss at the time of low water availability (Bartels and Sunkar 2005). ABA production by *A. brasilense* Sp245 was reported by Cohen et al. (2008), but like GA compounds, production mechanism is not known.

Polyamines

Polyamines are low molecular weight organic compounds containing two or more primary amino groups. These compounds are synthesized in cell, but their exact function is not clear. However, if synthesis of these compounds is inhibited, cell growth is stopped or inhibited. Among polyamines, cadaverine is known to promote root growth and control stomata activity in plants (Bashan and de-Bashan 2010). *A. brasilense* Az39, a common inoculant for wheat and maize in Argentina, is known to produce polyamines including spermidine, spermine, putrescine in culture, and cadaverine in growth medium supplemented with precursor L-lysine and in rice plants inoculated with this strain (Thuler et al. 2003; Perrig et al. 2007). Bashan et al. (2004) proposed cadaverine as a contributing factor in whole plant response due to *Azospirillum* inoculation.

Ethylene

Ethylene is known for breaking seed dormancy and inhibition of root elongation if produced in higher amount. Ethylene level is lowered to prevent economic loss in agriculture and can be considered as having potential for promoting plant growth (Glick et al. 1999). 1-Aminocyclopropane-1-carboxylic acid (ACC) is a common precursor of ethylene, and ACC deaminase degrades this precursor. Although ACC deaminase is very common among PGPR, wild strains of *Azospirillum* spp. do not have this enzyme; nevertheless, some strains can produce ethylene (Perrig et al. 2007). However, ACC deaminase gene (*acd* S) was recently detected in *A. lipoferum* 4B, and a homolog of *acd* S gene has been identified in *Azospirillum* B510 genome (Pringent-Combaret et al. 2008; Kaneko et al. 2010).

Nitrogen Oxide (NO)

NO functions as a signal molecule in IAA-induced signaling cascade leading to adventitious root development (Pagnussat et al. 2002, 2003).

The role of NO in lateral root formation and root hair development is demonstrated by Correa-Aragunde et al. (2004) and Lombardo et al. (2006). *Azospirillum* is known to produce NO at low oxygen pressure by denitrification, since a long time ago (Hartmann and Zimmer 1994). Creus et al. (2005) quantified the production of 6.4 nmol NO/g of *A. brasilense* growing under aerobic conditions.

Phosphate Solubilization

Microbes have the ability to solubilize insoluble phosphate by producing enzymes such as phytases and phosphatases or organic acids. Enzymes help to release organic phosphorus, and organic acids release inorganic phosphorous. Around 40 % of culturable bacteria from soil have this ability (Reis et al. 2011). It is not common among species of *Azospirillum*. However, *Azospirillum* spp. can produce different organic acids that assist in phosphorus solubilization depending on sugars in root exudates. There are few reports available about *A. halopraeferans*, *A. lipoferum*, and *A. brasilense* on this. Gluconic acid production and pH reduction were observed in *A. lipoferum* JA4. For *A. brasilense* strains, pH reduction and gluconic acid production were observed when carbon source of the medium was replaced by glucose (Rodriguez et al. 2004). *A. halopraeferans* solubilized inorganic phosphorous (Seshadri et al. 2000). A recent report about phosphate solubilization by *A. brasilense* WS-1 was published by Tahir et al. (2013).

Siderophore Production

Siderophores are low molecular weight iron chelating compounds which help beneficial bacteria to take away the ferric ion and make it unavailable to pathogenic bacteria. It is considered as indirect growth mechanism by which PGPR promote plant growth. Catechol-type siderophore production by *A. lipoferum* and *A. brasilense* was reported earlier by Shah et al. (1992) and Tapia-Hernandez et al. (1990). Recently, *Azospirillum* strains isolated from tomato rhizosphere had shown the production of catechol-type siderophores (Manivannan and Tholkappian 2013). Strains were not identified up to species level in this study. There are several reports for the siderophore production by *A. lipoferum* and *A. brasilense*. Boyer et al. (2008) detected siderophores in two strains TVV3 and B518 of *A. lipoferum*. Pedraza et al. (2007) isolated *A. brasilense* strains from strawberry plants and detected siderophore production by these strains. According to their observation, the amount of siderophore production has a correlation with its area of isolation, i.e., rhizosphere or within the plants. Endophytic strains produced higher amount.

Antimicrobial Activity

Oliveira and Drozdowicz (1987) reported bacteriocin production by *Azospirillum*. Somers et al. (2005) detected the production of Phenyl acetic acid (PAA) – an auxin-like molecule with antimicrobial activity, in culture supernatant, when phenylalanine was added as precursor. Tapia-Hernandez et al. (1990) isolated bacteriocin-producing *Azospirillum* strains that inhibited the growth of several other bacteria. In vitro HCN production by *Azospirillum* strains isolated from Brazil has been reported by Goncalves and de Oliveira (1998). Siderophores also exhibit antimicrobial activity against various bacterial and fungal isolates (Shah et al. 1992).

Azospirillum as a Biofertilizer

Plant Growth Promotion Due to Nitrogen Fixation

The oldest concept about plant growth promotion due to *Azospirillum* inoculation is based on its nitrogen-fixing ability. Although there are doubts about transfer of this fixed nitrogen to the plant, still several reports are available to support the contribution through BNF. A recent review by Okumura et al. (2013) discussed about BNF data for *Azospirillum*. The review is focused on five

crops and BNF contribution to these plants. Some of the examples selected from this review are described here. Brasil et al. (2005) inoculated a mixture of *A. brasilense* and *A. lipoferum* to *Brachiaria* plants and noticed higher root, shoot dry weights, and nitrogen content as compared to uninoculated plants. Kennedy et al. (2004) mentioned 26 % increase in corn productivity under field conditions. Saikia et al. (2007) suggested that high yield of *Azospirillum*-inoculated plants is due to higher photosynthesis and stomatal conductance in these plants. Okon and Vanderleyden (1997) reviewed 22-year field inoculation data of *Azospirillum* and concluded that this organism promotes crop productivity under different climates and soil conditions and goes beyond increase in BNF as increase in absorptive surface of root system was also observed. Sala et al. (2007) reported 26 % increase in wheat grain production when inoculated with *Azospirillum*. Diaz-Zorita and Fernandez-Canigia (2009) used liquid-based inoculants of *A. brasilense* for wheat, from 2002 to 2006, at 297 localities in Argentine Pampas region. The increase in productivity was 260 kg/ha and 70 % of grain yield. Several researchers are convinced that a combination of nitrogen fertilizer with *Azospirillum* inoculation is beneficial for wheat plants as inoculation promotes better absorption and utilization of available nitrogen. Dose of fertilizer recommended for combination varies from 15 to 160 kg N/ha (Saubidet et al. 2002; Dalla Santa et al. 2004; Sala et al. 2005).

Many studies showed that the contribution of nitrogen fixation by *Azospirillum* to the plant ranged from 5 to 18 % of total nitrogen increase in plant (Bashan and de-Bashan 2010). However, conclusive proof of the nitrogen contribution is based on ^{15}N dilution technique. Garcia de Salamone et al. (1997) used ^{15}N isotope dilution technique to determine the contribution of BNF to two genotypes of maize, inoculated with a mixture of *Azospirillum* strains. Significant increase in grain yield and total nitrogen content of both genotypes was observed. Saubidet and Barnex (1998) inoculated five cultivars of wheat with most efficient nitrogen-fixing strains of *A. brasilense*. Increase in growth and nitrogen content was observed, but response varied among cultivars. Didonet et al. (2003) inoculated ten lines of *Oryza sativa* with *A. brasilense* and *A. lipoferum* and reported that response depends upon cultivar and strains used. Mehnaz and Lazarovits (2006) reported significant increase in root and shoot dry weight of four corn varieties, inoculated with *A. zeae* (misidentified at that time as *A. lipoferum*); however, response varied with all varieties. Although *Azospirillum* is considered as a nonspecific PGPR with respect to host, there are several studies indicating that response varies among varieties and cultivars of the plants. Rodrigues et al. (2008) inoculated rice plants with *A. amazonense* and observed increased grain dry matter and nitrogen accumulation at maturation. Contribution from nitrogen fixation was up to 27 %. The comprehensive review by Bashan and de-Bashan (2010) based on the analysis of 35-year data suggested that even though there are controversial reports about nitrogen contribution to the plant due to nitrogen fixation by *Azospirillum*, the mechanism cannot be ignored.

Plant Growth Promotion due to Phytohormone Production

When plants are inoculated with bacteria, root system is the first plant part which shows response. Inoculation increases number and length of root hairs, number of lateral roots, diameter and length of lateral roots and adventitious roots, and root surface area. The increased root system can improve plant nutrition and in general is responsible for plant growth promotion. Change in the root system is considered due to the production of auxins, and *Azospirillum* is well known for these compounds. El-Khawas and Adachi (1999) inoculated rice plants growing in hydroponic system, with filter-sterilized supernatants of *Azospirillum* culture. Increase in root dry weight, surface area, lateral roots, root hairs, and root elongation as compared to uninoculated plants was observed. Cell-free supernatant of *A. brasilense* Cd was applied to soybean plants, and increase in root numbers and length was observed (Molla et al. 2001). In contrast, wheat inoculation

with *A. brasilense* Sp 245 and Sp7 decreased root length; however, root hair formation increased (Dobbelaere et al. 1999). This type of effect on root system is very common when higher concentrations of IAA are applied exogenously. Remans et al. (2008) proved that response of exogenously applied IAA on bean roots resembled to *Azospirillum* inoculation. Malhotra and Srivastava (2006) engineered *A. brasilense* SM strain and made it to produce threefold higher IAA as compared to wild-type strain. *Sorghum* plants inoculated with this strain showed higher effect on lateral branching and dry weight of roots when compared with plants inoculated with wild type. Although there are several reports of plant growth promotion due to IAA production by *Azospirillum*, considering it as a single mechanism responsible for growth promotion needs more evidences.

Cohen et al. (2009) inoculated *A. lipoferum* in maize plants which were unable to produce ABA and GA, due to application of inhibitors of biosynthetic pathways of these compounds. It causes the effect similar to drought; however, inoculation with *A. lipoferum* reversed the effect which suggested the participation of ABA and GA in alleviating water stress in the presence of the bacteria. So far, several researchers are convinced that water stress alleviation of plants by *Azospirillum* involves the production of ABA, auxins, and GAs (Costacurta and Vanderleyden 1995; Bottini et al. 2004; Cohen et al. 2008).

Plant Growth Promotion Due to Phosphate Solubilization

Among 15 species of *Azospirillum*, *A. halopraeferens* can solubilize insoluble inorganic phosphate in vitro by unknown mechanism as it does not use glucose, and therefore does not produce gluconic acid (Seshadri et al. 2000). Some strains of *A. brasilense* and *A. lipoferum* are capable of producing gluconic acid and can solubilize rock phosphate (Puente et al. 2004; Rodriguez et al. 2004). Chang and Li (1998) isolated *Azospirillum* strains from ectomycorrhizal sporocarp which degraded limestone, marble, and calcium phosphate in vitro. Carrillo et al. (2002) inoculated cardon (cactus) with *A. brasilense* Cd strain and reported enhanced phosphate solubilization and plant growth. Deubel et al. (2000) inoculated pea plants with *Azospirillum* strains and observed a shift in sugars under phosphorous deficiency which increased the capability of *Azospirillum* spp. to mobilize phosphate. Sugars such as glucose are part of pea root exudates grown in phosphorous-deficient soils, thus increasing the capacity of *Azospirillum* spp. to solubilize insoluble $Ca_3(PO_4)_2$. It was observed that under phosphorous deficiency glucose content decreased, while galactose, ribose, xylose, and fructose increased in root exudates.

Plant Growth Promotion by Minimizing the Stresses

The use of *Azospirillum* in addition to other PGPR to alleviate the salt stress and minimize the negative effects of several plant stresses including water stress has been recommended by several researchers (Bacilio et al. 2004; Bashan et al. 2004; Lucy et al. 2004). Salt resistance among *Azospirillum* species increased from *A. amazonense* (lowest) to *A. halopraeferans* (highest), as the latter tolerates over 3 % that is equivalent to seawater salinity (Bashan and de-Bashan 2010). *A. brasilense*-inoculated wheat plants showed a significant increase in shoot elongation, fresh and dry weights, water content, and relative water content in shoots, when grown under 320 mM NaCl and 20 or 30 % polyethylene glycol (PEG) 6000 causing salt or osmotic stress, respectively (Creus et al. 1997). Barassi et al. (2006) used *A. brasilense* Sp245 to inoculate lettuce seeds and observed the effect of salt stress. Higher total fresh and dry weights were recorded as compared to uninoculated plants. Inoculated dried seeds maintained the ability to mitigate the effect of salt on lettuce germination. The same strain alleviated the salt stress in carrot and chickpea as well (Hamaoui et al. 2001; Ayrault 2002). Rueda-Puente et al. (2004) suggested the use of *Klebsiella pneumoniae* and *A. halopraeferens* as auxiliary biofertilizers to improve the growth of *Salicornia bigelovii* (halophyte) in coastal

semiarid zones. Later on, the same group reported that yield loss due to stress diminished in *Azospirillum*-inoculated wheat plants, and grains showed 38.4, 22.2, and 125 % higher Mg, K, and Ca, respectively, as compared to uninoculated ones (Creus et al. 2004).

Inoculation with *Azospirillum* improved growth under water stress conditions, which was initially demonstrated in the 1980s (Bashan and Levanony 1990). Okon (1985) stressed in a review about the importance of *Azospirillum* in improving water status of the plants. It suggests that *Azospirillum* inoculation can protect the crops against drought. Sarig et al. (1990) inoculated *Sorghum bicolor* plants to osmotic stress in hydroponic system and observed that due to *A. brasilense* inoculation adverse effects such as reduction in leaf senescence were diminished. Corn plants inoculated with *Azospirillum* showed less effect of water stress on plant growth, as increase in root growth, total aerial biomass, foliar area, and proline content in leaves and roots was observed (Casanovas et al. 2002). *Azospirillum* inoculation alleviated the stress on wheat plants grown under drought conditions (El-Komy et al. 2003). Creus et al. (2004) inoculated wheat seeds with *A. brasilense* Sp245 at flowering stage and subjected the plants to drought. Significantly higher water content, relative water content, water potential, apoplastic water fraction, lower cell wall modulus of elasticity values, reduction in grain yield loss, and significantly higher Mg, K, and Ca in grains were observed in inoculated plants. Moutia et al. (2010) inoculated two cultivars of sugarcane with *Azospirillum* sp. strains under drought and non-drought conditions. Both cultivars showed different results as one showed 15 % increase in shoot height and 75 % increase in dry weight under drought, whereas the other cultivar showed negative results under non-drought conditions. Kasim et al. (2013) used *A. brasilense* NO40 to prime the wheat seeds and grew them under drought conditions. Priming significantly alleviated the deleterious effect of drought stress on wheat. Inoculated plants showed attenuated transcript level suggesting improved homeostatic mechanism due to priming.

Rodriguez-Salazar et al. (2009) used an engineered strain of *A. brasilense* with the ability to produce trehalose (osmotic regulating sugar), more salt tolerant than wild type, to inoculate maize plants grown under stress. Inoculation significantly enhanced survival of these plants under water stress. According to Bashan and de-Bashan (2010), limitations regarding the effect of inoculation under saline stress are valid for osmotic stress.

There are few reports about decreasing the toxic effect of metals on plants due to *Azospirillum* inoculation. Cadmium (Cd) inhibits the growth and nutrient uptake in barley plants. *A. lipoferum-inoculated* barley plants showed decreased toxicity to Cd, enhanced root length and biomass, and significant increase in amount of nutrients absorbed (Belimov and Dietz 2000; Belimov et al. 2004). Lyubun et al. (2006) observed less accumulation of arsenic in *A. brasilense* Sp245-inoculated wheat plants as compared to uninoculated plants. The association of *Azospirillum* changed the speciation, bioavailability, and uptake of arsenic by plants.

There are individual reports about positive effect of *Azospirillum* inoculation on plants growing under different stresses such as pH, toxic substances, high light intensities, humic acid, etc. High pH decreases the microalgal population. *A. brasilense* inoculation in microalgal culture eliminated the negative effect (de-Bashan et al. 2005). High level of tryptophan reduced multiplication of *Chlorella vulgaris*, but co-culturing with *A. brasilense* significantly reduced the inhibition (de-Bashan and Bashan 2008). Sometimes light intensity has negative effect on crops. Wheat inoculation with *A. brasilense* Cd significantly increased quantity of photosynthetic pigments chlorophyll a, b and auxiliary photoprotective pigments violaxanthin, zeaxanthin, antheraxanthin, lutein, neoxanthin, and ß-carotene that help the plant to sustain photosynthesis under unfavorable light conditions (Bashan et al. 2006). Hernandez et al. (2006) used *A. brasilense* to inoculate *Chlorella* spp. that is used for wastewater treatment and observed enhanced growth and metabolism of the microalgae. Bacilio et al. (2003) inoculated wheat seeds

with *A. brasilense* or *A. lipoferum* and observed improved seed germination and plant growth in soils amended with compost containing high concentration of humic acid. Both bacterial species survived in high humic acid solutions and modified compost composition "in vitro."

Considering the reports about *Azospirillum* effect on plant growth under different stresses, it is clear that several known and unknown mechanisms are involved in the interaction of this organism with plants. Based on these findings, Cassan et al. (2009b) and Sgroy et al. (2009) proposed a term "plant stress homeo-regulating bacteria (PSHRB)" for all rhizosphere PGPR/PGPB including *Azospirillum* that regulate homeostasis in plants under abiotic stresses.

Miscellaneous Effects of *Azospirillum* on Plants

Azospirillum inoculation in maize plants led to major quantitative and qualitative modification of secondary metabolites especially benzoxazinoids (Walker et al. 2011). *Azospirillum* produces nitric oxide (NO) which participates in metabolic, signaling, defense, and developmental pathways in plants and can also modify root architecture (Molina-Favero et al. 2007, 2008). *Azospirillum* can produce nitrite as part of its normal metabolism which also participates in plant growth promotion (Zimmer et al. 1988). Nitrate reductase (NR) activity of wheat leaves decreased by *Azospirillum* inoculation and increased in nitrate assimilation was also reported (Ferreira et al. 1987). Some of the lectins produced by *Azospirillum* induced changes in mitotic state of growing onion plant cells (Nikitina et al. 2004). Cell surface lectins isolated from *A. brasilense* affected activities of α-glucosidase, ß-glucosidase, and ß-galactosidase in the membrane and apoplast fractions of roots of wheat seedlings (Alenkina et al. 2006). *A. brasilense* Cd inoculation enhanced the proton efflux of wheat root (Bashan 1990). Inoculation of soybean seedlings with the same strain significantly reduced the membrane potential in every root part (Bashan 1991; Bashan and Levanony 1991).

Bashan et al. (1992) proved the effect of *A. brasilense* on root cell membrane of soybean and cowpea plants. Pereyra et al. (2006) showed that fatty acid composition of main phospholipids in roots is affected by *A. brasilense* inoculation. *A. lipoferum* 34H increased the phosphate ion content, resulting in significant increase in root length and fresh and dry weight of shoots of rice plants (Rodriguez and Fraga 1999). During dry storage, seeds deteriorate and germination gets delayed. Lettuce seeds inoculated with *A. brasilense* along or after an osmo-priming treatment could mitigate the negative effect of ageing (Carrozzi 2005).

Azospirillum as a Biocontrol Agent

The role of *Azospirillum* as a biocontrol agent has always been ignored, although reports for its anti-pathogenic activity were available since a long time ago. Mechanisms such as environmental competition, displacement of pathogens, inhibition of seed germination of parasitic weeds, inhibition of fungal growth via the production of toxic substances, and growth enhancement of plants to resist pathogen are suggested for *Azospirillum* to play its role as a biocontrol agent (Bashan and de-Bashan 2010).

As an Antimicrobial Agent

When *A. brasilense* Cd was co-cultured with pathogenic *Staphylococcus* spp., population of pathogenic bacteria got reduced (Holguin and Bashan 1996). Mixed cultures of *A. brasilense* and tomato pathogen *Pseudomonas syringae* were inoculated on tomato seeds. Significant decrease in severity of disease and improved plant growth was observed as *P. syringae* did not survive in the presence of *A. brasilense* (Bashan and de-Bashan 2002). *A. brasilense* reduced the dry weight of mycelium of four soilborne fungal pathogens of cucumber, i.e., *Fusarium* (90–96 %), *Rhizoctonia* (72–94 %), *Pythium* (71–95 %), and *Sclerotinia* (100 %), during an in vitro study (Hassouna et al. 1998). Sudhakar et al. (2000)

applied *A. brasilense* as foliar spray against foliar fungal and bacterial diseases of mulberry. Inoculation reduced fungal pathogens and excelled as a treatment against bacterial blight. Co-inoculation of *Azospirillum* spp. with arbuscular mycorrhizal (AM) suppressed damping off disease in *Capsicum* sp., caused by *Pythium aphanidermatum* (Kavitha et al. 2003). Tortora et al. (2011) isolated *A. brasilense* strains with the ability to produce catechol-type siderophores including salicylic acid. Inoculation of these strains reduced the anthracnose disease caused by *Colletotrichum acutatum* in strawberry plants.

As a Pesticide

Inoculation of *A. brasilense* to *Sorghum* resulted in tenfold decrease of dead heart disease caused by a shoot fly *Atherigona soccata* and also resulted in increase in grain yield (Kishore 1998). Only a few root galls and egg masses per root system, of mung bean plants infected with root-knot nematode, were observed when inoculated with *A. lipoferum* (Khan and Kounsar 2000). The increase in pod yield and reduction in root-knot nematode egg masses and total nematode population was observed in Okra plants, inoculated with *A. brasilense* (Ramakrishnan et al. 1997). A combination of mycorrhizal fungi, phosphate-solubilizing bacteria, and *Azospirillum* reduced the population of *Pratylenchus zeae* (nematode) and increased the cob yield of maize (Babu et al. 1998). *A. lipoferum* inoculation in wheat plants reduced *Heterodera avenae* (nematode) infection (Bansal et al. 1999).

As a Herbicide

Azospirillum spp. inhibited germination of a parasitic weed, *Striga hermonthica* (witch weed), of *Sorghum* fields (Bouillant et al. 1997). Lipophilic compounds produced by *A. brasilense* in culture medium inhibited the germination of *Striga* seeds (Miché et al. 2000). *A. brasilense* inhibited germination and radicle growth of *Orobanche aegyptiaca* (Egyptian broomrape) seeds, a parasitic weed of sunflower (Dadon et al. 2004).

As an Elicitor of Induced Systemic Resistance

The role of rhizobacterial exopolysaccharides (EPS) as elicitor of induced systemic resistance (ISR) was reported by Kyungseok et al. (2008). Recently, Sankari et al. (2011) used different concentrations of EPS produced by *Azospirillum* strains as inoculants of rice plants. EPS-inoculated plants showed better growth and reduction in blast disease caused by *Pyricularia oryzae. Azospirillum* sp. B510 induced disease resistance in rice plants against *Xanthomonas oryzae* and fungus *Magnaporthe oryzae*. Resistance was due to a novel mechanism independent of salicylic acid-mediated defense (Yasuda et al. 2009).

Azospirillum as a Commercial Biofertilizer

Azospirillum is capable of colonizing vegetables, cereals, and several other plants and induces beneficial effects as it congregates several plant growth-promoting mechanisms which make it a valuable candidate as a biofertilizer. For more than 40 years, researchers are using *Azospirillum* as an inoculant in lab, pots, and field experiments. Several reviews are available which have covered the 20–35-year data based on growth promotion by different strains of this genus. All of the researchers and reviewers agree on one point that this bacterium is beneficial for plant, and there is not a single report about its harmful effect. However, there is also an agreement that plant growth promotion is not due to any single mechanism; rather, it is a combination of several mechanisms possessed by *Azospirillum*, and in a recent review Bashan and de-Bashan (2010) proposed it as "multiple mechanisms theory." According to this theory "there is no single mechanism involved in promotion of plant growth by *Azospirillum* but a combination of a few or many mechanisms in each case of inoculation." These may vary according to plant species, *Azospirillum* strain, and environmental conditions when interaction occurred. Actually, possession of several mechanisms makes this organism one of the best candidates for bioinoculants.

Different countries have launched the *Azospirillum* as a biofertilizer for different crops. Information given below is based mainly on Reis et al. (2011) and Internet search:

1. *Azo-Green*™: A product of Genesis Turfs Forages Company, based in the USA, contains only *A. brasilense*. Recommended usage is as seed coat to improve seed germination, root system, drought resistance, and plant health.
2. *Zea-Nit*™: A product of Heligenetics Company, formulated in Italy, Germany, and Belgium, contains a mixture of *A. lipoferum* Br17 and *A. brasilense* Cd. It is sold as liquid formulation or in a mixture of vermiculite. Company recommended the usage of 30–40 % less nitrogen fertilizer with inoculum.
3. *Azo-Green*™: A French company launched a biofertilizer under the same name as used by the previously mentioned US company. However, this inoculant is specific for maize and contains *A. lipoferum* CRT-1. Based on field experiments carried out in Africa, 100 % increase in yield was claimed.
4. *Fertilizer for Maize*: A product developed by University of Puebla, Mexico, in 1993.
5. *Inoculant for coffee plants:* In 2008, *Azospirillum* inoculant was developed for coffee plants in Mexico.
6. *Graminante*™: A product of Uruguay contains bacterial inoculum in powder form, mixed with calcium carbonate.
7. *Inoculant for maize and rice*: In 2009, a company in Brazil started selling a biofertilizer based on *Azospirillum* strains for maize and rice.
8. *Inoculant for cereals and sunflower*: Several companies of Argentina are selling biofertilizers based on *A. brasilense* for cereal crops, sunflower, etc.
9. *Gmax Nitromax*: A product of GreenMax AgroTech, India, containing a mixture of *Azospirillum* and *Azotobacter*, available for all crops except legumes. There are several companies in India which are selling biofertilizers containing single or combinations of different microbes including PGPR and fungi.

BioPower ®
Adds Nitrogen and Improves Soil Fertility
PATENT BY GOVT. OF PAKISTAN
National Institute for Biotechnology & Genetic Engineering (NIBGE)

Fig. 15.4 A biofertilizer of *Azospirillum* for wheat crop, commercially available in Pakistan

10. *Bio N-Plus*: A product of Pakistan contains *A. lipoferum*.
11. *BioPower*: A product of Pakistan contains a mixture of PGPR including *A. brasilense* and *A. lipoferum* (Fig. 15.4).

Conclusion

Bashan and de-Bashan (2010) concluded three facts about *Azospirillum*: (1) Most *Azospirillum* strains can fix nitrogen but only a fraction of it is transferred to the plant; (2) many strains produce phytohormones "in vitro" and some in association with plants, but transfer is probably limited; and (3) a general positive growth response in numerous plant species is evident in majority cases, but effect is not always apparent in terms of economic productivity. If we look at these points, it reflects that although authors have suspicions about the contribution of nitrogen fixation and phytohormone in health of inoculated plants, they also report that they are convinced that inoculation does end up in plant growth promotion even if it is not always in terms of yield

increase. I would suggest that researchers should consider the importance of parameters other than yield increase because if we look from a farmer's point of view, then his concern is the plant growth, and *Azospirillum* does that by all means. However, from a researcher's point of view, it is important to find evidence of the claimed mechanisms which means a more comprehensive study of plant microbe interactions.

Going through the data available since *Azospirillum* was isolated for the first time, it is very clear that most of it is based on inoculation of *A. lipoferum* and *A. brasilense*. Similarly, if we look into details of *Azospirillum* biofertilizers available all over the world, these products also contain either *A. lipoferum* or *A. brasilense*, individually or together. Considering these facts:

(a) It is suggested that researchers should start exploring the rest of the 13 species for their potential as biofertilizers.
(b) As the importance of *Azospirillum* as a biocontrol agent has been always ignored, now it is time to focus on this aspect and study it as extensively as its role as a biofertilizer has been evaluated.
(c) Although several companies are selling *Azospirillum* as a biofertilizer, still it is not as popular as *Rhizobium* biofertilizer. The main reason is variable response of this organism with different crops which suggests that extensive optimization studies for the inoculum amount with reference of specific crops are still required.
(d) It is suggested that optimization studies should also be performed for different cultivars and varieties as enough reports are present to support that inoculation response varies with genotype of the same host.

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Ectomycorrhizal Fungi and Their Applications

16

Antonietta Mello, Elisa Zampieri, and Raffaella Balestrini

Abstract

Ectomycorrhizal (ECM) fungi form association with relatively small number of plants that dominate boreal, temperate, Mediterranean, and some subtropical forest ecosystems. These plant species have been able to acquire metabolic capabilities through symbioses with ECM fungi, thus improving their mineral nutrition and growth in several ecological niches. Mycorrhizal fungi can also play several other important ecological roles, including the protection of plants from abiotic and biotic stresses. Several "targeted" metagenomic projects have been carried out, or are now in progress, in order to identify the fungal communities in soil, including ECM fungi, which are present in various habitats (e.g., forest and truffle-ground soils, etc.). ECM fungi, which are important both because of their economic value as edible fungi (i.e., truffles, boletes) and because of their application in reforestation projects, are the subject of this chapter, in which the recent advances in ECM fungal communities are reviewed, focusing mainly on the applicative aspects related to the use of these fungi.

Introduction

Mycorrhizae are widespread symbiotic interactions between soil fungi and the roots of almost all land plants, including forest trees, grasses, and many crops (Smith and Read 2008). It has been estimated that about 90 % of terrestrial plant species undergo an improvement in mineral nutrient uptake, thanks to root symbiosis with mycorrhizal fungi (Brundrett 2009), which, in turn, provide the fungus with carbon compounds (i.e., sugars). Several mycorrhizal associations exist, and these are identified primarily on the basis of the taxonomic

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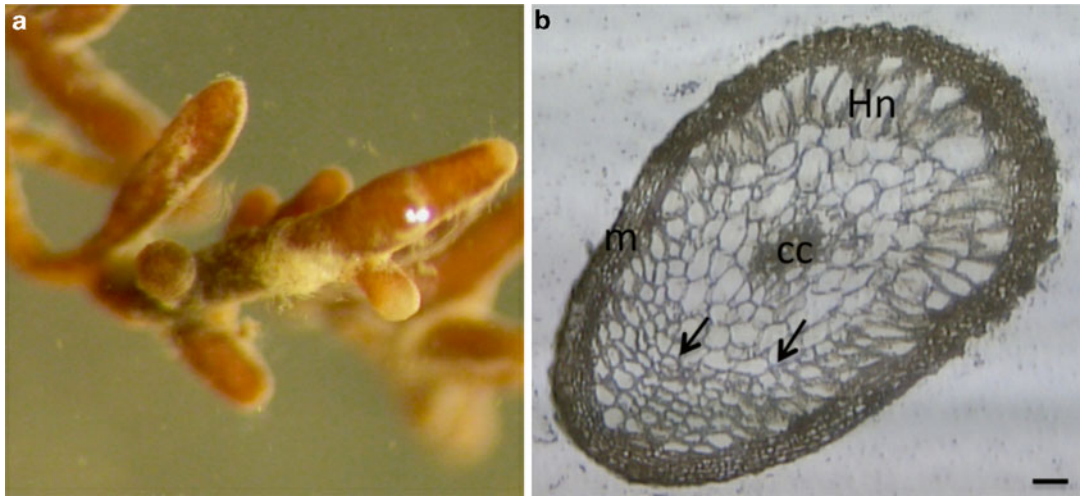

Fig. 16.1 (**a**) *Corylus avellana/Tuber melanosporum* ectomycorrhizae with the typical clavate aspect and the presence of external mycelium. (**b**) Paraffin oblique transverse section of a *C. avellana/T. melanosporum* ectomycorrhiza showing the mantle (m), which consists of several layers of hyphae, and the Hartig net proliferation (Hn and *arrows*). *cc* central cylinder. Bar corresponds to 25 μm

identity of the hosts and the structural features of the symbiotic interfaces (Smith and Read 2008). According to the ability of the mycorrhizal fungus to penetrate the root cells, mycorrhizae can be divided into two main types: endomycorrhizae and ectomycorrhizae (Balestrini et al. 2012; Perotto et al. 2013).

In ECM symbiosis, which is the subject of this review, the fungus forms a hyphal sheath, called mantle, which is made of aggregated hyphae that surround the root surface, and the Hartig net, which is formed by the hyphae that penetrate between the root cells (Balestrini et al. 2012; Fig. 16.1). The mantle is thought to be responsible for the mineral nutrition and water uptake of the symbiotic tissues, while the Hartig net is considered the site in which metabolites are exchanged. A preliminary confirmation of the functional diversity of these two fungal compartments can be found in the study on *Amanita muscaria* ECMs by Nehls et al. (2001) in which the separation of the mantle from the ECM root, using only tweezers, underlined a differential expression for two hexose-regulated fungal genes. A specificity in the mantle and Hartig net transcriptomic profiles, which reflects a functional specificity, has recently been revealed by Hacquard et al. (2013) through a laser microdissection approach, in which the two fungal compartments in truffle ECM were dissected, in combination with microarray gene expression analysis.

ECM fungi occur all over the world, and their host range includes most angiosperm and gymnosperm trees, as well as economically important timber-producing tree species. They play an important ecological role in woodland and forest communities in boreal, temperate, Mediterranean, and some subtropical forest ecosystems (Tedersoo et al. 2010a) that are dominated by hundreds of ECM fungal species (i.e., *Basidio-* and *Ascomycetes*), which form symbiotic associations with the lateral roots of trees and shrubs (Bonfante 2010). ECM symbiosis involves a relatively small number of plants, i.e., those belonging to Betulaceae, Dipterocarpaceae, Fagaceae, Nothofagaceae, Myrtaceae (e.g., *Eucalyptus*), Pinaceae, Salicaceae, and some genera of shrubs (e.g., Cistaceae), which are assisted by ECM fungi in the nutrition and protection against root diseases (Smith and Read 2008). As far as the fungal part is concerned, the majority of ECM fungi belong to *Basidiomycetes*, which can form macroscopic epigeous fruiting bodies that often grow next to tree trunks in woodlands, such as *Boletus edulis* and *Cantharellus cibarius*, while some others belong to *Ascomycetes* and form hypogeous fruiting bodies, such as truffles (Girlanda et al. 2007).

ECM fungi in the soil are highly competitive for nutrient acquisition (Bücking et al. 2012 and reference therein), and their colonization allows plant access to N forms that are not so easily available to non-mycorrhizal plants (Hobbie and Hogberg 2012 and reference therein). In line with these observations, Averill et al. (2014) and the relative commentary by Bradford (2014) have suggested that the presence of greater stores of organic matter in forest soils dominated by ECM fungi, than in those dominated by arbuscular mycorrhizal (AM) fungi, could be due to a reduced nitrogen availability for the free-living microbes that use organic matter. Interestingly, when a tripartite interaction is present, as in the *Alnus* host system (N-fixing bacteria, ECM fungi, *Alnus* roots), the ECM fungal activity seems to be shifted toward a greater capacity for organic P acquisition (Walker et al. 2013). A study about the impact of an ECM fungus (*Hebeloma cylindrosporum*) on the potassium (K^+) nutrition of its host plant (*Pinus pinaster*) has recently been performed, and the involvement of a fungal K^+ transporter has been investigated (Garcia et al. 2014). The results have shown that the K^+ nutrition of mycorrhizal pine plants was significantly improved under potassium-limiting conditions (Garcia et al. 2014).

A positive correlation between ECM symbiosis and plant performance in drought conditions has been reported (Morte et al. 2001; Dunabeitia et al. 2004; Alvarez et al. 2009), although plant response to drought can vary depending on the ECM fungal species (Dosskey et al. 1991; Kennedy and Peay 2007). More recently, a pot experiment, using *Pinus sylvestris* seedlings inoculated with several ECM fungi (*Cenococcum geophilum*, *Paxillus involutus*, *Rhizopogon roseolus*, and *Suillus granulatus*), has shown that only *S. granulatus* has a positive effect on shoot growth. Two different watering regimes (moist *versus* dry) were considered, and it was shown that *S. granulatus* effect on shoot growth was more pronounced under moist conditions (threefold increase) than under dry conditions (twofold increase), thus suggesting that the considered ECM fungus did not provide any additional support during drought stress (Kipfer et al. 2012). Danielsen and Polle (2014) have instead investigated the nutrient status and the physiological responses to drought of young poplar trees in the presence/absence of the ECM fungus *P. involutus*, and they have been shown that root tips from ECM plants have a higher vitality than those from non-mycorrhizal plants. Since this effect is evident in both the colonized and non-colonized tips of ECM plants, the authors have suggested that it could be due to a general improved water supply to the roots of the host plants.

Numerous *in situ* $^{13}CO_2$ pulse-labeling experiments have been conducted on annual crops or grasslands to demonstrate a rapid carbon flux pathway from the host to the roots and from the roots to the rhizosphere (Robin et al. 1990; Nguyen et al. 1999; Johnson et al. 2002; Leake et al. 2006). The studies on carbon allocation in trees using pulse labeling have usually been performed in microcosms or mesocosms (Norton et al. 1990; Ek 1997; Simard et al. 1997), and only a few studies have been conducted in situ with adult trees but have never considered the fruiting bodies of the associated fungi (Högberg et al. 2008; Plain et al. 2009; Subke et al. 2009; Epron et al. 2011). Recently, Le Tacon et al. (2013) have elegantly assessed the allocation of carbon by the host to *Tuber melanosporum* mycorrhizae and ascocarps via an *in situ* $^{13}CO_2$ pulse-labeling experiment performed on a 20-year-old hazel tree in a truffle orchard established in the northeast of France. Almost all of the carbon allocated to the truffle ascocarps came from the host; thus, the hypothesis that it was mainly supplied via saprotrophic pathways was excluded. The development of truffles requires that carbon is stored in the trunk or roots of the host, and the process takes several weeks/months unlike what happens in an ectomycorrhizal member of *Basidiomycotina* producing fruiting bodies over a number of days (Teramoto et al. 2012). This result, as expected, demonstrates that the processes involved in carbon acquisition and ascocarp development are different from those of basidiocarps, since *Tuber* ascocarps take at least 6 months to grow between the production of the primordia and full ascocarp development, unlike *Basidiomycota* sporocarps, which develop over a number of days directly from diploid mycorrhizae.

ECM symbiosis can therefore be considered a crucial component in nutrient cycling in sustainable forest ecosystem, and the current genome sequencing projects on ECM fungi are providing useful information to understand the functional and ecological roles for these fungi (Martin et al. 2008, 2010; Martin and Nehls 2009; Plett and Martin 2011; Martin and Bonito 2012; Balestrini et al. 2013; Marmeisse et al. 2013).

ECM fungi, which are therefore important for both their economic value (i.e., truffles, boletes) and their use in reforestation projects, are the subject of this chapter, in which the recent advances in ECM fungal communities are reviewed, focusing mainly on the applicative aspects related to the use of these fungi.

Research on ECM Fungi: From the Past to the Present

The study of ECM fungal diversity initially focused on the screening and molecular identification of fruiting bodies and, only later, on ECM tips, usually after sorting these in morphotypes (Horton and Bruns 2001; Mello et al. 2006a). As each ECM species is specialized in exploiting specific resources of the soil ecosystem, investigations have focused on the spatial distribution of the extraradical mycelium. Tracking the distribution of a given ECM fungus is considered difficult, since fruiting bodies do not reflect the distribution of ground networks (Dahlberg 2001). *H. cylindrosporum* was the first ECM fungus to be detected in soil (Guidot et al. 2002), and its presence was revealed within 50 cm from the fruiting bodies. Thanks to the use of the β-tubulin gene as a marker, Zampieri et al. (2010) were able to show that *T. magnatum* mycelium is more widespread than could be inferred from the distribution of its fruiting bodies and ECM and were able to identify a new haplotype that had never been described before from fruiting body material. The application of the denaturing gradient gel electrophoresis (DGGE) technique made it possible to discover that *T. melanosporum* is the dominant fungus in an area characterized by scanty vegetation, known as brûlé, which is associated to this fungus, and that *Basidiomycota* ECM fungi decrease within the brûlé, thus indicating a competitive effect of *T. melanosporum* on other ECM fungi (Napoli et al. 2010).

Since each individual within a species has its own functional traits, the next step toward a better understanding of the role of biodiversity in ecosystem functioning will need to consider the intraspecific diversity of mycorrhizal plants and fungi (Johnson et al. 2012).

The possibility of studying (micro)organisms directly in the field (metagenomics or environmental genomics, Chivian et al. 2008) thanks to the introduction of high-throughput sequencing techniques (i.e., 454 pyrosequencing) has given a strong impulse to the development of projects devoted to the study of fungal communities in different environments, including soils. The first studies on fungal diversity, which used a metagenomic approach in combination with high-throughput technology, appeared in 2009 (Buée et al. 2009; Jumpponen and Jones 2009). Buée and colleagues investigated fungal diversity in six different forest soils from a temperate French site using tag-encoded 454 pyrosequencing of the ITS-1 (nuclear ribosomal internal transcribed spacer-1), while Jumpponen and Jones (2009) studied the fungal communities in leaves of *Quercus macrocarpa* of trees located inside and outside a small urban center using the same molecular target. These authors have demonstrated that 454 pyrosequencing can be used successfully to study fungal communities in forest soil and phyllosphere. Starting from these first works, an approach based on 454 GS-FLX pyrosequencing has been widely used to investigate fungal communities in soils and has allowed new information to be provided on ECM fungal communities in several biomes/ecosystems, e.g., Swedish spruce plantations (Wallander et al. 2010); tropical African forests (Tedersoo et al. 2010b); truffle grounds (Mello et al. 2011); transgenic poplar plantations (soils and roots; Danielsen et al. 2012); ECM herb *Bistorta vivipara* roots on the Arctic archipelago of Svalbard (Blaalid et al. 2012, 2014); an urban landscape

(Lothamer et al. 2013); a boreal forest (Clemmensen et al. 2013); boreal and tropical forests (McGuire et al. 2013); truffle grounds, a Mediterranean agro-silvo-pastoral system, serpentine substrates, and a contaminated industrial area (Orgiazzi et al. 2013); an oak-dominated forest in Japan (Toju et al. 2013); and three microsites (decayed wood, mineral soil adjacent to intact logs, control mineral soil) in mature spruce forests in British Columbia (Walker et al. 2014).

Global climate change is supposed to influence soil fungal communities, including ECM fungal communities. It has already been demonstrated that several ecological factors, disturbances (e.g., fire; Kipfer et al. 2011), and management practices (e.g., nitrogen deposition; Peter et al. 2001) affect the composition of ECM fungal communities and can lead to a reduction in the number of ECM fungal species (Koide et al. 2011). Changes in ECM fungal communities have recently been investigated using 454 pyrosequencing. Voříšková et al. (2014) have shown that seasonality and soil depth can influence ECM fungal communities in a temperate oak forest soil. A decrease in ECM fungi abundance has been verified in buried soils in the Siberian tundra (Gittel et al. 2013), where the abiotic conditions (low temperature and anoxia) seem to favor an abundance of bacteria, facultative anaerobic decomposers of soil organic matter (SOM) such as *Actinobacteria*, which increase compared to unburied soils. Hui et al. (2011) have observed, in a boreal coniferous forest site in Southern Finland, that a long-term exposition to Pb contamination can lead to a shift in the composition of the ECM community associated with the dominant pine (*P. sylvestris* L.), as well as an increase in the abundance of the OTUs (operation taxonomic units) assigned to the *Thelephora* genus and a decrease in the frequency of OTUs corresponding to *Pseudotomentella*, *Suillus*, and *Tylospora* in the contaminated zone (Hui et al. 2011).

However, although several factors influence the composition of an ECM community and species richness of ECM fungi, the functional consequences of these shifts on the aboveground communities (e.g., for tree performance), as well as for the soil ecosystems, still require further investigation (Kipfer et al. 2012). An extensive metabolic reprogramming during the colonization between *Laccaria bicolor* and its compatible host *Populus trichocarpa* has recently been demonstrated. However, this extensive metabolic reprogramming is repressed in incompatible interactions where more defensive compounds are produced or retained (Tschaplinski et al. 2014). Moreover, Pena and Polle (2014) have demonstrated, using ^{15}N isotope enrichment, that ECM assemblages provide advantages for inorganic N uptake mainly under environmental constraints with respect to unstressed plants, thus suggesting a stress activation of specific ECM taxa.

In addition to their ecological role, some ECM fungi such as *Tricholoma matsutake*, the so-called pine mushroom, have great value as commercial food. This mushroom is popular in Asia because of its aromatic odor and particular taste, as well as its high nutritional and medicinal value (Ohnuma et al. 2000; Kim et al. 2008; Ding et al. 2010). However, its annual production is limited, and several abiotic factors (e.g., rainfall and temperature) can affect it; moreover, attempts to cultivate it artificially have been unsuccessful. Kim and colleagues (2013) have investigated, through a 454 GS-FLX pyrosequencing platform, the fungal communities in soil where fruiting bodies develop. Several zones have been considered (inside, beneath, and outside the fairy ring zone of *T. matsutake*), with the aim of obtaining information on the fungal communities that could influence the development of the fruiting bodies. Mello et al. (2011) have used a similar approach to verify the fungal populations inside and outside the brûlé (burnt area), in which *T. melanosporum* fruiting bodies (the black truffles) are usually collected. The results show that *Ascomycota*, which was the most dominant phylum in the investigated French truffle ground, are more abundant inside than outside the brûlé, while *Basidiomycota* increase outside the brûlé in agreement with previous results reported by Napoli et al. (2010) using the DDGE technique.

The Application of ECM Fungi to Field Projects

ECM fungi provide trees with several benefits, such as the enhanced ability to absorb water, phosphorus, and nitrogen and protection from soilborne root pathogens such as *Fusarium oxysporum*. Evidence of the positive role of ECM *Pisolithus albus* in enhancing the growth of plants, such as *Acacia spirorbis* and *Eucalyptus globulus*, has been observed by significant increases in shoot and root biomass and mineral nutrition (P, K, and Ca), as well as a limited metal uptake, acting as a protective barrier, in nickel-rich ultramafic topsoils in New Caledonia (Jourand et al. 2014). In the restoration of ultramafic ecosystems degraded by mining activities, it could be convenient to isolate indigenous and stress-adapted beneficial ECM fungi in order to inoculate endemic plants (Jourand et al. 2014). ECM fungi have already been proposed for the ecological restoration of mine sites (in particular chromium and nickel mines) in Australia by Reddell et al. (1999) and in New Caledonia by Perrier et al. (2006) as well as in the post-mining of bauxite in Brazil by Khosla and Reddy (2008). ECM fungi are also expected to play a key role in forest regeneration after major disturbance events such as stand-replacing forest fires. Kipfer et al. (2010) investigated the heat tolerance of ECM fungi of Scots pine. They verified that 60 and 70 °C reduced the mean of the species, but not 45 °C. The composition changed because of heat, but most of the ECM fungi, such as *R. roseolus*, *C. geophilum*, and several unidentified species, survived.

Sustainable soil ecosystem services require the management of the beneficial soil organisms that are considered of economic value and which are available in the market for their application. Reforestation using container-grown seedlings of *P. pinaster* produced in nurseries is a common practice in many countries. Fertilizers are often used in nurseries. However, the use of chemical fertilizers can constitute a threat to the environment, in addition to modifying nutrient availability of the fertilized seedlings, which may not be able to adapt to forest soil conditions when transplanted. Sousa et al. (2012) showed that selected ECM fungi could be used as a beneficial biotechnological tool in the nursery production of *P. pinaster*, without the need of fertilizers. Oliveira et al. (2012) found that inoculation with selected ECM fungi can be an advantageous ecotechnological approach that can be used to improve the nursery production of *P. pinaster*. The inoculation of Chinese pine (*Pinus tabulaeformis* Carr.) seedlings with *Boletus luridus*, under field experimental conditions, has shown to significantly influence bacterial functional diversity in the rhizosphere of *P. tabulaeformis* seedlings, thus highlighting the importance of the application of ECM fungal inoculum in order to promote microbial community diversity of soil in forest restoration projects (Zhang et al. 2010).

The fast growth rate of American chestnut, coupled with its quality timber, makes it a desired species for use in reforestation projects. Bauman et al. (2013) have evaluated various soil preparation methods that promote ECM colonization and American chestnut *Castanea dentata* establishment in coal mine restoration projects. *Quercus ilex* forests play ecological and socioeconomic roles by protecting the environment and providing wood, forage resources, and tourism. Oliveira et al. (2010) considered the management of nursery practices for efficient ECM fungi application in order to establish *Q. ilex* plantations. The ECM inoculants could be rhizosphere soil from forests, spores, or vegetative mycelia, although the soil from forests has the disadvantage of perhaps containing both beneficial (e.g., mycorrhizal fungi) and harmful (pathogenic fungi) microorganisms. The use of spores or vegetative mycelia of ECM fungi seems to be the most convenient and practical technique although the supply of spores is limited by the onset of the rainy season and the maturing of the fruiting bodies. Aggangan et al. (2012) have developed a protocol for the production of quality rooted cuttings for plantation establishment or enrichment planting in the red soil of Caliraya, Philippines, of *Anisoptera thurifera* (Blanco) Blume and *Shorea guiso* (Blanco) Blume, which belong to the *Dipterocarpaceae*, the most important tree family in the tropical forests of southeast Asia, and are considered as endangered species. These authors have shown

that ECM mycelia entrapped in alginate beads are effective in promoting the growth and P uptake of *A. thurifera* and *S. guiso*. Immobilized mycelium offers more advantages than non-immobilized inoculum such as longer survival in the soil, easy storage, and greater viability. However, the cultivation in fermenters or in bioreactors for the large-scale production of mycelial inoculum may compromise the quality of the inoculum and thus limit the application of this technique. Forecasting the response of ECM fungi to environmental changes represents an important step in maintaining forest productivity for the future. Jarvis et al. (2013) have analyzed and identified fungal communities from 15 semi-natural Scots pine (*P. sylvestris* L.) forests through ITS sequencing. Their data have demonstrated an important effect of rainfall and soil moisture on community composition at the species level and less influence of temperature on the abundance of ECM exploration types. Valdés et al. (2006) observed that a severe drought had an important effect on both total fine-root biomass and the ECM-root biomass in a tropical pine forest and suggested that forest management practices should consider the effects of drought in reducing the capacity of *Pinus oaxacana* to form ECM.

Food Applications and Toxicity of ECM Fungi

Fungi are the most productive biological sources of primary and secondary metabolites that have long been exploited by the pharmaceutical and food industries. Some of them, such as *Boletus* species, are also an important source of proteins, carbohydrates, fatty acids (mainly linoleic acid), sugars (mainly mannitol and trehalose), and vitamins (tocopherols and ascorbic acid), as well as phenolic acids (Heleno et al. 2011). Since phenolic acids have antioxidative properties, they are currently being exploited by food and pharmaceutical industries. Important antioxidative properties have been found for polysaccharides extracted from *B. edulis*, and these could be employed as ingredients in healthy food to alleviate oxidative stress (Zhang et al. 2011). A lectin with antitumoral properties has also been found, thus opening new perspectives in research, with the aim of developing new drugs for cancer therapies (Bovi et al. 2011). A homologous of the sugar-binding antiviral protein cyanovirin-N (CVN), previously identified in the cyanobacterium *Nostoc ellipsosporum*, has been found by analysis of transcript sequences deriving from a gene expression profiling study conducted in the truffle *Tuber borchii*, and a novel protein family has been described in filamentous fungi and in the fern *Ceratopteris richardii* (Percudani et al. 2005). It has been proposed that these findings provide candidate polypeptides to be tested as antiviral agents (Percudani et al. 2005). Two genes coding for putative lectins belonging to the CVNH (CyanoVirin-N Homolog) family have also been found in *T. melanosporum* genome (http://mycor.nancy.inra.fr/IMGC/TuberGenome/index.html). However, preliminary results have shown that TbCVNH (the CVNH discovered in *T. borchii*) seems to be completely inactive in the antiviral activity test (Koharudin et al. 2008). Mushrooms produce particular aroma compounds that are of interest for industrial applications, such as 1-octen-3-ol, which is often added as flavoring to processed products in order to reintegrate its loss which occurs during the preparation of these food products (Zawirska-Wojtasiak 2004). Long-chain unsaturated fatty acids, such as palmitoleic acid and linoleic acid, show antibacterial activity and are used as antimicrobial food additives (Zheng et al. 2005). To date, more than 200 volatile organic compounds have been described from various truffle species, and the biosynthetic pathways involved in volatile biosynthesis have been traced in the genome of a mushroom that is highly appreciated for its special taste and aroma, the black truffle *T. melanosporum* (Martin et al. 2010). From the ecological point of view, truffle volatiles are used to attract mammals and insects, which are thus able to locate the precious hypogeous fungi, feed on them, and spread their spores. In addition, truffle volatiles diffuse in the soil and mediate complex interactions with microorganisms and plant roots (Splivallo et al. 2011; Mello et al. 2013). Truffles and porcini are greatly appreciated throughout the world, both as fresh fruiting bodies and as ingredients in processed products (Mello 2012).

A qPCR assay has been developed to authenticate and quantify *T. magnatum* and *T. melanosporum* in food matrices that have undergone intensive transformation processes, such as cream and butter (Rizzello et al. 2012). This method is promising in detecting the incorrect labeling of processed products and can therefore be used to protect the consumer and to assess food quality. The rapid identification of mushroom poisoning, which continues to be a public health concern in Europe, the USA, and several other parts of the world, is particularly important. The majority of reported fatal intoxications have been attributed to a few species of the *Amanita* genus and in particular to the death cap *Amanita phalloides*, which can cause a high mortality rate (10–30 % in adults). *A. phalloides* are often mistaken in appearance for nonpoisonous species, and the ingestion of one single mushroom cap may be sufficient to cause death within 2–8 days (Gausterer et al. 2014). Besides the previous publications that have reported the use of conventional and real-time PCR in the cases of suspected mushroom poisoning as an alternative to morphological investigations and as a complementary approach to toxicological analyses, an article on a rapid and sensitive detection of genetic traces from poisonous mushrooms in a variety of matrices, including raw, fried, and digested mushroom homogenates, spiked feces, and clinical samples (vomit, stool), has just been published by Gausterer et al. (2014). Mushrooms belonging to the *Boletus edulis* sensu lato group, a complex of at least four species of ECM fungi in the genus *Boletus* section *Boletus* (Singer 1986), constitute an interesting example of contrasting fungal features. These fruiting bodies are in fact in high demand as mushrooms because of their pleasant flavor and texture, but at the same time, they have been shown to induce allergic symptoms either through inhalation, ingestion, or contact (Helbling et al. 2002). In order to guarantee safe naturally derived food, Mello et al. (2006b) developed specific primers for the unambiguous detection of *B. edulis* sensu stricto, *B. aereus*, *B. pinophilus*, and *B. aestivalis*. In addition, the relationships of *B. violaceofuscus* with the members of *B. edulis* s.l. have been examined. The data that will be obtained from the *B. edulis* genome sequencing project, which is currently in progress, will surely allow our knowledge on its allergenic potential and the presence of allergen orthologues to be improved. Thanks to the availability of the genome sequence, an extremely low allergenic potential and the lack of key mycotoxin biosynthetic enzymes have been found in the black truffle *T. melanosporum* (Martin et al. 2010).

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Increasing the Role of Mycorrhizal Symbiosis in Plant-Plant Facilitation Process to Improve the Productivity and Sustainability of Mediterranean Agrosystems

17

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Abstract

Plant-plant facilitation is an ecological process occurring in most terrestrial ecosystems. Plant-plant facilitation is considered as a positive interaction between both plant partners in which one plant species promotes the growth, survival or reproduction of the neighbouring plant. Recent studies have underlined the role of mycorrhizal fungi, i.e. arbuscular mycorrhizal fungi (AMF) and particularly extraradical hyphae of AMF, in interconnecting plants and consequently their importance in plant-plant facilitation process. Networks of AMF impact soils both physically and biologically and are considered as an important pathway for the transference of nutrients such as nitrogen and phosphorus. In parallel,

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AMF increase plant growth and nutrient uptake and decrease the deleterious effects of pathogens and drought. The aims of this chapter are to describe how the AMF are involved in the plant facilitation process and to assess the main mycorrhizal effects on the plant nutrition (P and N) and plant health. Also the contribution of AMF in cropping systems as well as agricultural strategies which improve AM associations in arid agrosystems will be reviewed and illustrated by experimental results from field studies in Mediterranean environment.

Introduction

In arid and semiarid environments, desertification of land area has increased in size over the last decades due to drastic climatic conditions (long periods of drought, erratic rainfall) combined with human activities (overexploitation of natural resources, grazing, etc.) (Francis and Thornes 1990). Desertification is commonly related to an alteration of the plant cover (species diversity, abundance), but it also impacts the physical, chemical and biological characteristics of soils (soil structure, nutrient bioavailability, microbial activity, etc.) (Garcia et al. 1997; Requena et al. 2001). Indeed, due to the exposure of soils to wind and water erosion, genetic and functional diversity of soil microorganisms is particularly affected by these biotic and abiotic degradations. Among the microbial components particularly susceptible to desertification, the diversity and abundance of mycorrhizal fungi are significantly reduced (Bethlenfalvay and Schüepp 1994). Mycorrhizal fungi play a major role in the dynamic of terrestrial plant ecosystems (Boucher et al. 1982). In addition to their direct impact on the development of the plant mainly by improving the mineral nutrition of the host plant, it has been shown that the structure of the plant cover and its development were closely linked to the functioning of mycorrhizal symbiosis (Odum 1959; van der Heijden et al. 1998). The ability of a plant species to tolerate biotic or abiotic stresses is also highly dependent on the degree of establishment and functioning of the symbiotic relationship between the fungus and the host plant (Odum 1959; Barea et al. 1997). It is also now well demonstrated that mycorrhizal symbiosis affects microbial soil functioning via its influence on some microbial groups involved in the major biogeochemical cycles (N, P and C) (Frey-Klett et al. 2005). Figure 17.1 depicts the various positive impacts of AMF on the plants and ecosystems as a whole.

Since the availability of mineral nutrients and the increase in abiotic and biotic stresses will become important limiting factors for plant production, the integration of key processes from natural ecosystems in agricultural management has been suggested as one strategy to overcome the increasing agricultural problems. In many cases, the non-degraded ecosystems governed by

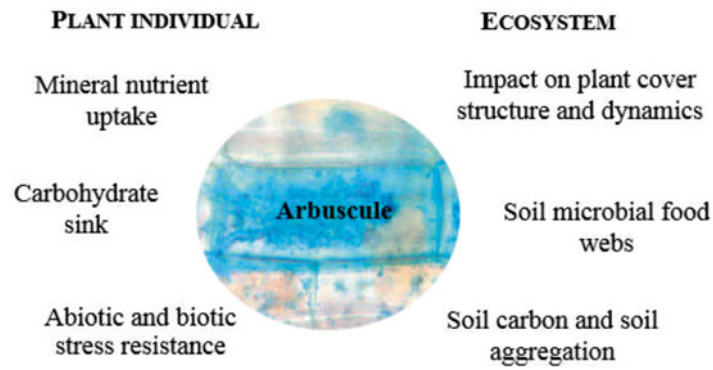

Fig. 17.1 AM symbiosis effects on plant and ecosystem (adapted from Fester and Sawers (2014))

Table 17.1 Key roles of AM symbiosis as an ecosystem service provider (modified from Gianinazzi et al. 2010)

AM function	Ecosystem service	References
Development of a complex, ramifying fungal network	Improvement of soil structure/soil stability/water retention	Bedini et al. (2009)
Root morphology modifications		
Enhancement of plant mineral nutrition and plant water uptake	Plant growth promotion with reduced phosphate fertiliser	Smith and Read (2008)
Secretion by AM fungi of "sticky" proteinaceous substances (glomalin)	Improvement of soil stability and water retention	Rillig et al. (2002)
Increase of crop plant tolerance against abiotic stresses (salinity, drought, etc.)	Improvement of plant resistance to root pathogens (nematodes, pathogenic fungi)	Marulanda et al. (2006)
Plant protection against biotic stresses	Improvement of plant resistance to drought, salinity, heavy metals, pollution, mineral nutrient deficiencies	Hao et al. (2009)
Activation of plant secondary metabolism	Improvement of plant quality (nutritional, medicinal, etc.) for human health	Gianinazzi et al. 2010

these natural processes that usually result from long periods of evolution are highly productive, resistant to pests and retentive of nutrients (Ewel 1999). AMF symbioses show particular potentialities with regard to plant stress resistance and mineral nutrient uptake and have key roles as ecosystem service provider (Gianinazzi et al. 2010) (Table 17.1). The mycorrhizal efficiency has been usually studied as the result of dual interactions between the host plant and the fungal symbiont. But it must be reconsidered as the fungal effect on the ecosystem derived from complex interactions with other microorganisms within the mycorrhizosphere (Bonfante and Anca 2009).

Among natural processes that are involved in the stability and the productivity of non-disturbed ecosystems, plant-plant facilitation could be considered as a powerful tool to sustain the mycorrhizal efficiency in agricultural conditions. Plant-plant facilitation is an ecological process occurring in communities worldwide (Valiente-Banuet et al. 2006) when the presence of one plant enhances the growth, survival and reproduction of its neighbour (Callaway 2007). The maintenance of plant-plant facilitation over time shows that the benefits of this association are not restricted to germination and seedling establishment but are extended to growth and long-term survival (Montesinos-Navarro et al. 2012). Plant facilitation has been experimentally described to be species specific (Castillo et al. 2010). Hence, the benefactor plant species (nurse plant) may promote the development of specific beneficiary (facilitated) species more strongly than others (Valiente-Banuet and Verdu 2007, 2008; Castillo et al. 2010). The main mechanisms involved in the facilitation processes, provided by the nurse plants, are: (i) microclimate improvement beneath their canopies (Gomez-Aparicio et al. 2004); (ii) protection from herbivores (Baraza et al. 2006); and (iii) enhancement of physical, chemical and biological soil properties (Puerta-Pinero et al. 2006). Some studies have suggested that mycorrhizal fungi could be a key ecological component to understand plant-plant facilitation processes (Van der Heijden and Horton 2009; Van der Putten 2009). Mycorrhizal fungi can interconnect plant individuals from different species or genera in natural communities (Newman 1988). These links between plants provide pathways for the transfer of major mineral nutrients such as N (He et al. 2005) and P (Smith et al. 2001).

All these scientific data show that it would be possible to introduce in agricultural practices some "nurse plants" providing mycorrhizal propagules for the benefit of the cultivated plant species (beneficiary plant species).

The Contribution of Mycorrhizal Symbiosis to Plant Nutrition and Plant Health

The Mycorrhizal Symbiosis and the Crop Nutrition

The most important benefit that AMF provide to their host plant is generally attributed to an enhancement of plant P uptake (Graham 2000). AM fungi can play an important role in crop P nutrition,

increasing total uptake and sometimes P use efficiency (Koide et al. 2000) leading in some cases to a growth and yield enhancement (Koide et al. 2000). The extraradical hyphae are important to the plants for acquisition of phosphorus (P) and other mineral nutrients (Read and Perez-Moreno 2003). There are examples where no response of crops to colonisation by native AMF was recorded (Ryan et al. 2002). This is generally attributed to a high concentration of available soil P (Sorensen et al. 2005).

Nitrogen (N) is a major nutrient that frequently limits primary productivity in terrestrial ecosystems. AMF could cause changes in N-cycling processes through five general pathways: (i) substrate availability (NO_3), (ii) modification of the abiotic soil environment (improved aeration through macroaggregate formation), (iii) microbial community shift (the influence of the hyphal exudation structuring the hyphosphere microbial communities), (iv) individual host plant level effect and (v) plant community shifts (effect of the plant cover composition). AMF assimilate N predominantly in the form of NH_4^+ (Tanaka and Yano 2006). Moreover, it is largely admitted that AMF can mobilise N from organic sources (Barrett et al. 2011). AMF are also important in the plant acquisition of other nutrients such as zinc (Zn), calcium (Ca), copper (Cu), etc. (Clark and Zeto 2000).

It has been frequently reported that AMF were the main actors in the P nutrition and N uptake enhancement. Other soil microorganisms, e.g. N-fixing bacteria or P-solubilising bacteria, may synergistically interact with AMF and thereby benefit plant development and growth (Puppi et al. 1994). Bacteria associated with the AMF can also assist in the uptake of nutrients from soil. Numerous examples of this are available from bacterial-AM-legume tripartite symbiotic relationships, where diazotrophic bacteria provide fixed N not only for the plant but also for the fungus. Nodulation of legumes by N-fixing bacteria and establishment of AMF often occur simultaneously and synergistically. Genes for N fixation in endosymbiotic *Burkholderia* bacteria have been detected in AM hyphae (Minerdi et al. 2001) suggesting potentialities for improved N supply to mycorrhizal plants through fixation of atmospheric N.

The Mycorrhizal Effects Against Plant Pathogens (Nematodes, Phytoparasitic Plants)

Large quantities of pesticides are usually used to limit the spread of pests that enforce great yield losses. However, these chemical products are detrimental to human health and to the environment, and in addition, they are often partially effective against soilborne diseases. Numerous studies have shown the beneficial effects of AMF in acting against the plant pathogens and in increasing the plant tolerance to these biotic stresses. Most of these researches have been carried out in controlled conditions at early stages of plant growth. In these conditions, it has been demonstrated that AMF exerted antagonistic effects against pathogenic fungi such as *Fusarium*, *Rhizoctonia*, *Verticillium*, *Phytophthora*, *Pythium*, etc. (Whipps 2004) (Table 17.2). The same fungal influences have been recorded on different genera of plant-parasitic nematodes such as *Heterodera*, *Meloidogyne*, *Pratylenchus* and *Radopholus* (Hao et al. 2009) (Table 17.2).

Management of the Soil Mycorrhizal Potential in Mediterranean Cropping System

The adoption of the plant-plant facilitation process for agricultural management has to be adapted to the edaphic conditions encountered in the cultural area. Two main approaches could be followed according to the soil nutrient contents and soil biological diversity: to adapt the process to current agroecosystems (the "reductionist" approach) or to adapt agroecosystems to fit the process in question (the "holistic" approach) (Fester and Sawers 2014) (Fig. 17.2).

The reductionist approach is based on the criteria of the "controlled mycorrhization" practice and is realised by using a selected AM strain adapted to specific conditions and compatible with the target crop, whereas the objectives of the "holistic approach" are to maximise fungal diversity for ensuring AMF-dependant ecosystem services. Hence, the reductionist approach

Table 17.2 Examples of pathogenic fungi and plant-parasitic nematodes controlled by AM fungi (adapted from Gosling et al. 2006)

Pathogen	Diseases	Crop	References
Sclerotium cepivorum	White rot	Onion	Torres-Barragan et al. (1996)
Fusarium oxysporum	Fusarium root rot	Asparagus	Matsubara et al. (2002)
		French bean	Dar et al. (1997)
		Tomato	Datnoff et al. (1995)
Verticillium dahliae	Verticillium wilt	Tomato	Karagiannidis et al. (2002)
		Aubergine	Matsubara et al. (2000)
Rhizoctonia solani	Root and stem rots	Mung bean	Kjoller and Rosendahl (1996)
		Tomato	Berta et al. (2005)
Helicobasidium mompa	Violet root rot	Asparagus	Kasiamdari et al. (2002)
Aphanomyces euteiches	Root rot	Pea	Bodker et al. (2002)
Phytophthora parasitica	Root rots	Tomato	Cordier et al. (1996)
Meloidogyne incognita	Root-knot	Tomato	Talavera et al. (2001)

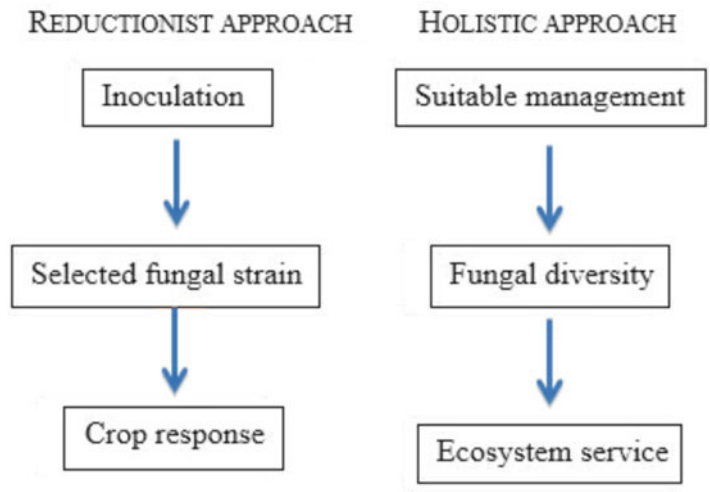

Fig. 17.2 Approaches to promote AM symbiosis for agronomic applications

will be encouraged to promote plant performance under low diversity circumstances. In contrast, the holistic approach will be implemented to conserve or restore native AMF diversity. In order to promote the AM fungal effect on plant growth through the management of the plant-plant facilitation process, some highly mycotrophic plant species (shrubs, tree) could be integrated in intercropping system to play the role of mycorrhizal propagule provider. Moreover and depending on the level of chemical fertility and AMF diversity, these nurse plants could be inoculated by selected AMF strains (reductionist strategy) or not inoculated but highly colonised by native AMF communities. For instance, the use of leguminous shrubs and trees as symbiotic islands under arid conditions could be beneficial for the AM propagule multiplication (Camargo-Ricalde and Dhillion 2003).

Experimental Results

The grain legume faba bean is cultivated worldwide as a protein source for food and feed. In addition, faba bean provides ecosystem services such as renewable inputs of nitrogen (N) into crops and soil resulting from biological N_2 fixation and a diversification of cropping systems (Tables 17.3 and 17.4). In some parts of the world

Table 17.3 Impact of faba bean intercropping on plant height, leaf area index (LAI), biomass accumulation and N uptake of three durum wheat varieties at time of faba bean incorporation (from Tosti and Guidicci (2010))

	Plant height (m)		LAI (m^2 m^{-2})		Biomass (Mg ha^{-1})		N uptake (kg ha^{-1})		N concentration (N)	
	2005	2006	2005	2006	2005	2006	2005	2006	2005	2006
Means of varieties										
Cappelli	0.28	0.30	1.04	1.64	1.22	1.59	18.5	24.7	1.53	1.57
Creso	0.21	0.25	0.82	1.13	0.98	1.23	17.5	20.7	1.80	1.65
Duilio	0.23	0.25	0.74	1.11	0.95	1.14	15.5	18.7	1.62	1.65
Means of techniques										
Intercropping	0.26	0.30	0.70	1.42	0.79	1.35	12.9	22.4	1.66	1.68
Sole crop	0.22	0.22	1.04	1.16	1.30	1.29	21.5	20.3	1.64	1.57
SEM										
Varieties	0.008*	0.015^{ns}	0.051*	0.04**	0.07^{ns}	0.063*	1.19^{ns}	2.28^{ns}	0.118^{ns}	0.100^{ns}
Techniques	0.006**	0.008**	0.040**	0.057*	0.068*	0.058^{ns}	1.12**	1.20^{ns}	0.055^{ns}	0.059^{ns}

Pooled standard error of the means (SEM) and significance of F test for varieties and techniques are reported. The variety × technique interaction was never significant so means of varieties (over techniques) and techniques (over varieties) are reported

ns not significant

*F significant for $P=0.05$, **F significant for $P=0.01$

Table 17.4 Impact of faba bean intercropping on plant height, dry biomass accumulation, plant N uptake, grain N accumulation and nitrogen harvest index (NHI, % of plant allocated to the grain) recorded at maturity in three durum wheat varieties (from Tosti and Guidicci (2010))

	Plant height (m)		Biomass (Mg ha^{-1})		N uptake (kg ha^{-1})		Grain N (kg ha^{-1})		NHI (%)	
	2005	2006	2005	2006	2005	2006	2005	2006	2005	2006
Means of varieties										
Cappelli	1.09	1.06	6.79	8.36	63.0	60.0	45.0	39.8	71.2	66.6
Creso	0.63	0.61	5.18	5.71	57.9	50.8	43.9	40.6	76.1	79.5
Duilio	0.63	0.64	4.81	6.34	51.8	64.9	43.3	52.6	83.0	80.8
Means of techniques										
Intercropping	0.80	0.84	5.50	7.25	63.2	66.4	49.5	51.4	78.6	77.4
Sole crop	0.77	0.71	5.68	6.35	51.9	50.6	38.6	37.3	74.9	73.8
SEM										
Varieties	0.022**	0.030**	0.368*	0.554^{ns}	4.29^{ns}	5.60^{ns}	4.10^{ns}	3.93^{ns}	2.79^{ns}	1.83**
Techniques	0.017^{ns}	0.024**	0.398^{ns}	0.329^{ns}	3.66^{ns}	2.67*	2.65*	1.67*	0.61*	0.56**

Pooled standard error of the means (SEM) and significance of F test for varieties and techniques are reported. The variety × technique interaction was never significant so means of varieties (over techniques) and techniques (over varieties) are reported

ns not significant

* F significant for $P=0.05$, ** F significant for $P=0.01$

(e.g. China), intercropping of faba bean with maize and other cereals is a common practice (Li et al. 2009). In this context, it has been reported that nitrogen sources from N_2 fixation and soil will be uptaken more efficiently (Li et al. 2009; Tosti and Guidicci 2010). Intercropping of faba bean with cereals appeared as an efficient management tool to control weeds and plant diseases (Hauggaard-Nielsen et al. 2008). Planting the cereal with faba bean will ensure earlier canopy closure and soil cover. It has also been shown that legume parasitic weed broomrape can be controlled by intercropping faba bean with cereals (Fernandez-Aparicio et al. 2007). Recently, it has been demonstrated that faba bean, cocultured with wheat in Morocco, could significantly enhance the mycorrhizal soil infectivity which explains all the beneficial effects recorded from previous studies (unpublished data). Hence, these interactions between the leguminous plant species (e.g. faba bean) and the cereal could be interpreted as a plant-plant facilitation interaction where faba bean plays the role of the nurse plant (benefactor plant species) species, whereas the other crop plant (e.g. wheat, maize, etc.) is the beneficiary (facilitated), and the facilitation is mediated by mycorrhizal fungi.

Conclusion

AMF offer an environmentally sustainable approach to increase crop production and health. In order to improve the performances of AM symbiosis on plant growth, the introduction of nurse plants in cultural practices (conventional or alternative agricultural regimes) could be a useful biological tool to sustainably introduce the fungal symbionts into the agricultural areas. However, major research efforts will be necessary to identify some biological parameters that will be discriminant for the choice of the AM fungal management strategy (reductionist vs. holistic strategies). In addition, it will be necessary to refine the techniques for detecting and following AMF strains or communities in the field.

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Role of Plant Growth-Promoting Rhizobacteria and Their Formulation in Biocontrol of Plant Diseases

18

S.S. Shaikh and R.Z. Sayyed

Abstract

Fungal phytopathogens are a major threat to crop plants. More than 60 fungal phytopathogens are known to cause various plant diseases. These diseases lead to the loss of 30 % crop yield that accounts to about 20,000 crore rupees per year in the global market. Different methods of chemotherapy have been used to control these diseases. However, uncontrolled use of chemical fungicides has severely affected the agroecosystem, leading to the development of pesticide-resistant pests and emergence of new species of pests. Increased dependence on chemicals is inevitably associated with environmental and health hazards. In this regard, plant growth-promoting rhizobacteria (PGPR) have been found as an effective, eco-friendly, and sustainable replacement to the chemical fungicides. The present chapter focuses on antifungal traits such as siderophores, lytic enzymes, hydrogen cyanide, and antibiotics of PGPR, development of effective formulation of PGPR for longer shelf life and competitiveness with the natural environment, obstacles in formulation development, and characteristics of bioformulation. Biotechnological attributes of PGPR with respect to plant growth promotion, disease resistance, and iron nutrition are also discussed.

Introduction

The world population is continuously increasing and it is estimated to reach ~8 billion by 2020. There is a real challenge to feed this huge population, an endeavor that requires agricultural productivity to be increased. Thus, more than ever, obtaining high yields is the main challenge for agriculture. In addition, in recent years, both producers and consumers are increasingly focusing on health and quality of food and on their organoleptic and

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nutritional properties. Fungal diseases of crop plants have led to the development of micronutrient-deficient food. Consumption of such staple food has led to micronutrient-deficient anemia, for instance, iron deficiency anemia (IDA). PGPRs are often seen as potential candidates that provide various micronutrients to crop plants by solubilizations, which decrease micronutrient deficiencies. PGPRs have been used as effective bioinoculants, biocontrol agents, mineral solubilizers, etc. In spite of various useful activities of PGPR, they fail to perform these functions under natural field conditions. A formulation of PGPR for sustained release and longer survival may improve their performance under field condition.

Plant Growth-Promoting Rhizobacteria (PGPR)

The term PGPR derived by Kloepper et al. (1980) is a group of plant-beneficial rhizobacteria, potentially useful for stimulating plant growth and increasing crop yields. In the last few years, the number of PGPRs has been found to increase mainly due to their role in the rhizosphere. Various species of bacteria such as *Pseudomonas*, *Azospirillum*, *Azotobacter*, *Klebsiella*, *Enterobacter*, *Alcaligenes*, *Arthrobacter*, *Burkholderia*, *Bacillus*, and *Serratia* have been reported to enhance the plant growth and suppress phytopathogens (Sayyed and Chincholkar 2009; Sayyed and Patel 2011; Sayyed et al. 2012, 2013; Saharan and Nehra 2011).

Bashan and Holguin (1998) proposed the division of PGPR into two classes: biocontrol plant growth-promoting bacteria (biocontrol PGPB) and plant growth-promoting bacteria (PGPB). This classification may include beneficial bacteria that are not rhizosphere bacteria, but this classification system has not been widely accepted. PGPR are known to provide essential nutrients for plant growth or to enhance their availability, to play an important role in pathogen suppression, and to improve the health of soil in the long term (Vessey 2003; Lucy et al. 2004; Lugtenberg and Kamilova 2009), and therefore are potentially important in reducing the use of chemical fertilizers and pesticides.

Biotechnological Attributes of PGPR

Plant Growth Promotion

PGPR increases the plant growth by nitrogen fixation (Kloepper et al. 1989), production of phytohormones (Gutierrez et al. 2001; Patten and Glick 1996), production of siderophore (Sayyed and Chincholkar 2009; Sayyed and Patel 2011), lowering of ethylene concentration (Madhaiyan et al 2007; Chinnadurai et al. 2009), and solubilization of phosphorous and various other minerals (Sayyed et al. 2007b; Sharma et al. 2013a).

Iron Nutrition in Crops

Iron is the fourth most abundant and common element present in the soil. It is an essential element for growth, metabolism, and survival of the majority of cell types on the Earth. It is an essential component of different processes involved in the biosynthetic pathways and in the formation of chlorophyll (Lopez-Millan et al. 2001), but it is rarely found in free form (Höfte 1993). Thus, it remains unavailable for crops even in iron-rich soils (Podile and Kishore 2006). When the availability of iron is inadequate for the growth of crops, leaves become pale green, yellow, or white in color, and eventually brown indicating iron deficiency (Brittenham 1994). Iron deficiency is a common nutritional disorder in many crop plants, resulting in poor yields and iron-deficient staple crops. The application of siderophore-producing PGPR is known to provide iron nutrition and to help in iron uptake by plants (Podile and Kishore 2006). Most microorganisms have developed efficient methods of absorbing iron from the environment and many microorganisms secrete siderophores to mobilize iron (Hider and Kong 2010). Once Fe (III) is mobilized and captured by the secreted siderophore molecule, it is accessible for cellular uptake by the plants. Sharma et al. (2013b) have shown that treatment of rice plants with siderophore-producing PGPRs results in increased iron content of the plants. Treatments with *Pseudomonas putida* B 17 and B 19 almost doubled the iron content of the plants.

Plant Disease Control

Biological control of plant diseases using antagonistic PGPR offers a highly effective, economical, and environment-friendly alternative to the use of synthetic pesticides (Emmert and Handelsman 1999). Plant disease suppression by soil microorganisms is the best possible alternative of reducing the chemical input in agriculture (Compant et al. 2005). Rhizobacteria are effective competitors in the rhizosphere, which can establish and persist on the roots of plants that are agronomically important.

The mode of action of the antagonistic organisms against various soilborne plant pathogenic fungi includes biosynthesis of antibiotics, production of hydrolytic enzymes, synthesis of cell wall lytic enzymes (Kim et al. 2003), production of siderophore, competition for substrates and the sites on the roots, and induced systemic resistance (ISR) (Ramamoorthy et al. 2001; Nakkeeran et al. 2004). Successful bacterial antagonists often show a synergistic combination of mechanisms responsible for a successful antifungal interaction.

Fluorescent pseudomonads and closely related species are important antagonistic bacteria present in the soil. Some of the fluorescent pseudomonads have currently received worldwide attention due to the production of a wide range of antifungal compounds, viz., fluorescent pigments, siderophores, volatile compounds such as hydrocyanic acid (HCN), antibiotics, and lytic enzymes (Sullivan and Gara 1992). Lytic enzymes (chitinase, β-1,3-glucanase, and protease) of PGPR are responsible for the lysis and hyperparasitism of antagonists against deleterious fungal pathogens. Such bacterial strains have been implicated in the plant growth as well as in the inhibition of plant pathogenic fungi and deleterious rhizobacteria showing a significant increase in root colonization. These attributes make PGPR an effective biocontrol agent (Garbeva et al. 2004; Ran et al 2005).

Some PGPRs do not produce metabolites against the pathogens, but still protect the plant from the disease, due to alteration of host defense mechanism accounting for the protection of observed disease. This mechanism includes ISR or systemic acquired resistance (SAR). ISR/SAR is defined as the activation of chemical and physical defense mechanism of the host plant by an inducer that can be a chemical or a microorganism, leading to the control of several pathogens (Kloepper et al. 1992) (Fig. 18.1).

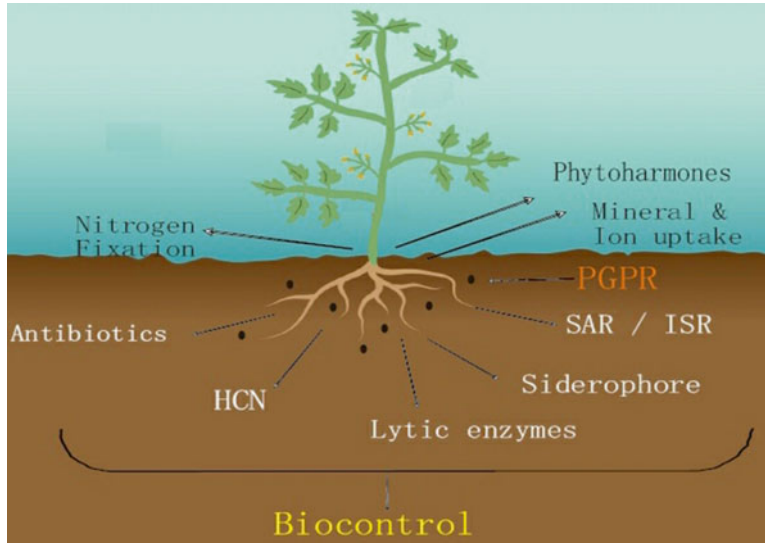

Fig. 18.1 Mechanism of action of PGPR including biocontrol

Antifungal Metabolites of PGPR

Siderophores

A siderophore (Greek word for iron carrier) is a low molecular weight (500–1,000 daltons), high-affinity, ferric, ion-chelating compound secreted by organisms. Under iron stress conditions, some microorganisms produce siderophores that chelate the available iron and prevent the iron nutrition of respective phytopathogen, and thereby restrict the proliferation and root colonization by the phytopathogen (Chaiharn et al. 2009; Miethke and Marahiel 2007; Sayyed and Chincholkar 2009). Siderophore-producing rhizobacteria are also known to impart ISR to plants (Pieterse et al. 2001) and suppressiveness to the soil (Mazzola 2002) and have been implicated in the biocontrol of several plant diseases (Sayyed et al. 2005).

Siderophore-producing PGPRs function as biocontrol agents by depriving the pathogen from iron nutrition (Lemanceau and Albouvette 1993) (Fig. 18.2). Siderophore-producing rhizobacteria rapidly colonize the plant roots of several crops and secrete an array of antifungal metabolites (Hass and Defago 2005). Chaiharn et al. (2009) studied the suppression of rice pathogen by siderophore-producing isolates. Gupta et al. (2002) studied the suppression of *Macrophomina phaseolina* causing charcoal rot of peanut by *Pseudomonas*. Freitas and Pizzinato (1997) have reported the inhibition of *Colletotrichum gossypii* by siderophore-producing rhizobacteria. Sindhu et al. (1997) reviewed the role of PGPR in the inhibition of phytopathogens. Johri et al. (1997) have reported the role of siderophore-producing fluorescent *Pseudomonas* strain RBT 13 that exhibited antagonistic action against several bacterial and fungal plant pathogens. Akhtar and Siddiqui (2009) reported that siderophore-producing pseudomonad significantly reduced the root rot disease in chickpea. However, microorganisms having the ability to produce powerful siderophores can only become ecologically competent biological control agents (BCAs) provided that they exhibit strong root-colonizing potential (Chincholkar et al. 2000).

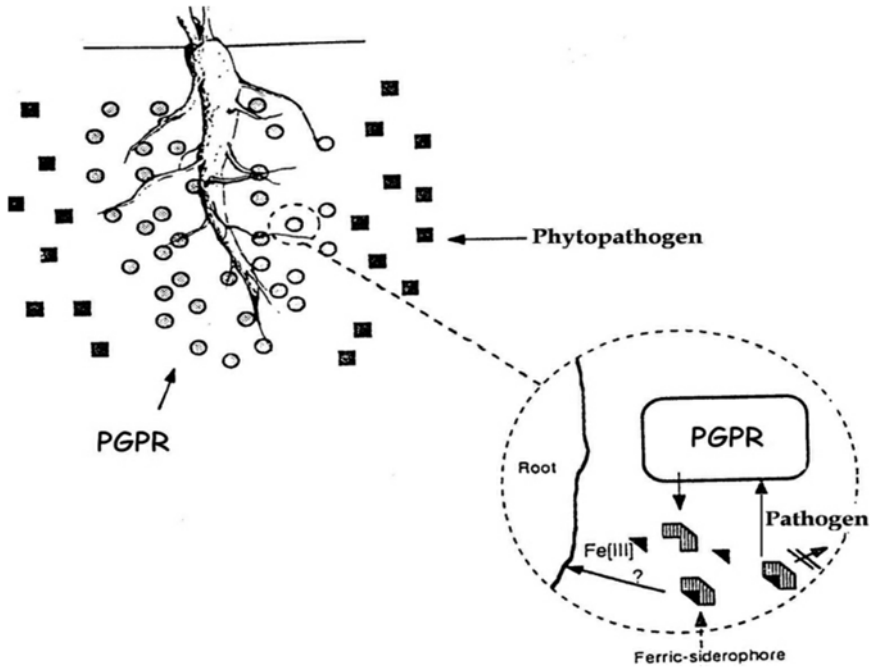

Fig. 18.2 Suppression of phytopathogens by siderophore-producing PGPR

Siderophore-based BCAs are gaining commercial significance because they are safer, their self-replication circumvents the need for repeated application, they do not lead to biomagnification, and the target organisms do not develop pesticide resistance (Sayyed et al. 2005). They also provide iron nutrition to roots, and thereby promote plant growth (Sayyed et al. 2007a).

Lytic Enzymes

Some PGPR strains have been found to produce enzymes that can lyse the cell wall of phytopathogenic fungi. Cell walls of most of the phytopathogenic fungi (except oomycetes) are made up of chitin $(C_8H_{13}O_5N)_n$, an unbranched, long-chain polymer of glucose derivatives, composed of β-1,4-linked units of the amino sugar N-acetyl-D-glucosamine (NAG). Chitinases (EC 3.2.1.14) are hydrolytic enzymes that break down glycosidic bonds in chitin (Kim et al. 2003). Thus, chitinases play a vital role in the biological control of many plant diseases by degrading the chitin polymer in the cell walls of fungal pathogens. It affects fungal growth through the lysis of cell walls, hyphal tips, and germ tubes. Chitinase is an inducible enzyme secreted by many microorganisms in the cultures containing chitin or its oligomers as sole carbon source. Chitinolytic enzymes have been reported from *Streptomyces* (Kim et al. 2003), marine isolates (Park et al. 2000), isolates from alkaline soil (Loni et al. 2011), and rhizosphere (Kishore and Pande 2007; Idris et al. 2007; Kamil et al. 2007). These microorganisms have been used as BCAs because the chitinases are involved in the antagonistic and antifungal actions against many phytopathogenic fungi (Singh et al. 1999; Frankowski et al. 2001; Gohel et al. 2005; Kim et al. 2008; Ramos-Solano et al. 2010). Velusamy and Kim (2011) showed chitinolytic activity of *Enterobacter* species against fungal hyphae. Some biocontrol strains produce enzymes including chitinases, cellulases, β-1,3 glucanases, proteases, and lipases that can lyse a portion of the cell wall of many pathogenic fungi (Glick 2012).

Hydrogen Cyanide

Hydrogen cyanide (HCN) is a secondary metabolite of some bacteria (Schippers et al. 1990). It is a colorless or pale blue liquid or gas with a faint, bitter, almondlike odor. It is a highly toxic, volatile compound that interferes with the cellular respiration by inhibiting the cytochrome oxidase enzyme in the mitochondria. This, in turn, prevents the production of ATP (adenosine-5′-triphosphate), the molecule that transfers energy in the cell. Thus, the cell is deprived of energy, and as a result, its growth is suppressed. It helps in the suppression of aerobic root pathogens (Schippers et al. 1990). The production of HCN requires glycine as a precursor (Castric 1977). Voisard et al. (1989) found that HCN-producing fluorescent *Pseudomonas* strain CHAO has the ability to suppress black root rot of tobacco, while HCN mutant of the wild-type strain of CHAO had lost its ability. HCN production in the rhizosphere has different effects on different plant types; still the role of HCN production in the control of root pathogens is unclear.

The detection of HCN in rhizosphere microbes has been described by many workers (Lorck 1948; Castric 1975; Askeland and Morrison 1983). The HCN produced by Gram-negative *Pseudomonas fluorescens*, *P. aeruginosa*, and *Chromobacterium violaceum* (Askeland and Morrison 1983) is detected by observing the change in color (from yellow to brownish red) of the indicator paper, amended with picric acid solution.

Antibiotics

Some well-known PGPR biocontrol strains such as *Pseudomonas*, *Bacillus*, *Azospirillum*, *Rhizobium*, and *Serratia* species are involved in the production of antibiotics such as phenazine-1-carboxylic acid, 2,4-diacetylphloroglucinol (DAPG), oomycin, pyoluteorin, pyrrolnitrin, kanosamine, zwittermicin A, and pantocin (Fernando et al. 2005). These antibiotics have been reported to be involved in the suppression of different pathogens by inducing fungistasis,

Table 18.1 Role of antifungal metabolites of PGPR in biocontrol of phytopathogens

AFM	Producing PGPR	Target pathogen	Crop
Siderophore	*P. fluorescence*	*Erwinia carotovora*	Potato
		G. graminis	Wheat
		Fusarium glycinia	Wheat
		Sarocladium oryzae	Soybean
	P. putida	*Fusarium* sp. wilt	Radish, cucumber
		Fusarium solani	Beans
		Erwinia carotovora	Potato
	P. cepacia	*F. oxysporum*	Onion
	P. aureofaciens	*G. graminis* var. *tritici*	Wheat
	B. pumilus	*G. graminis* var. *tritici*	Wheat
	Enterobacter aerogenes	*P. cactorum*	Apple
	Bradyrhizobium sp.	*F. solani*	Sunflower
		R. solani	Mung bean
	Rhizobium meliloti	*M. phaseolina*	Groundnut
Chitinase	*Enterobacter* spp.	*Aphanomyces* and *Pythium*	Pea
		Botrytis	Apple
	Streptomycetes	*S. sclerotiorum*	Potato, tomato
	Bacillus thuringiensis	*R. solani*	Cotton
		All phytopathogens	
	Pseudomonas sp.	*Fusarium* sp.	Banana
		M. phaseolina	Maize, payn
HCN	*P. fluorescence*	*Thielaviopsis basicola*	Tobacco, wheat
		G. graminis	
Antibiotics	*P. fluorescens* (DAPG)	*G. graminis tritici*	Take-all disease
		Thielaviopsis basicola	Tobacco
		P. ultimum	Sugar beet
	P. cepacia	*Bipolaris maydis*	Maize
	(Pyrrolnitrin)		
	P. fluorescence	*Sclerotinia homoeocarpa*	Grass
	(Pyoluteorin)	*Pythium* sp.	Cucumber

inhibition of spore germination, lysis of fungal mycelia, or exerting fungicidal effects (Fravel 1988). Cronin et al. (1997) have reported that DAPG produced by *Pseudomonas* species is responsible for suppressing phytopathogens. It was demonstrated that with increasing populations of *Pseudomonas* species producing the antibiotic DAPG, there was a rapid decline in take-all disease in wheat caused by the fungus *Gaeumannomyces graminis* var. *tritici* (de Souza et al. 2003). Several strains of *Bacillus subtilis* produce iturin (Yu et al. 2002; Chitarra et al. 2003) bacillomycin D (Besson and Michel 1992), plipastatins, surfactin, and aminopolyol that exert strong antimicrobial action against *Pythium ultimum*, *Rhizoctonia solani*, *Fusarium oxysporum*, *Sclerotinia sclerotiorum*, and *M. phaseolina* (Constantinescu 2001).

Phenazine, a potent antibiotic produced by *P. fluorescens*, has been used to control take-all disease of wheat caused by *G. graminis*. *Agrobacterium radiobacter*, a first commercially applied BCA for controlling crown gall in dicots, produces agrocin, which specifically inhibits *Agrobacterium tumefaciens* (Sindhu et al. 1997). Table 18.1 shows the role of AFMs of PGPR in the biocontrol of phytopathogens. Genetic analysis of many biocontrol strains of *Pseudomonas* indicated that there is a positive correlation between disease suppression and antibiotic production (Vincent et al. 1991).

Development of Effective Formulation

Though biocontrol with PGPR is an acceptable green approach, the registration of biocontrol agents for commercial availability is very low. Handling these products requires care, but these are not properly handled by the people who use it. This is the reason that there are so many PGPRs that perform very well in controlled conditions but not in field conditions, and hence the commercialization of these products is not easy. Since bioformulation is a living product, utmost care is needed at all the steps beginning from the production till the end use to maintain the microbial load and vigor. In addition, the present-day bioproducts can be further improved to obtain greater efficiency levels. The development of formulations with increased shelf life and broad spectrum of action with consistent performance under field conditions can pave the way for commercialization of the technology at a faster rate.

In general, biofertilizers (rhizobacteria) are living microorganisms. Unlike chemical fertilizers, they themselves are not the source of nutrient, but can help the plants in accessing the nutrients available in its surrounding environment (Table 18.2). The development of a successful inoculant involves several critical elements such as strain selection, selection of a carrier, mass multiplication, formulation of the inoculant, and packaging and marketing. The functions of formulations include stabilizing the microorganism, aiding in its delivery, protecting the microbe at the target zone, and enhancing the functionality of the microbe against the target (if a BCA).

Characteristics of PGPR and Their Formulation

To develop a successful PGPR formulation, rhizobacteria should possess certain characteristics such as high rhizosphere competence; high competitive saprophytic ability; enhanced plant growth; ease for mass multiplication; broad spectrum of action; excellent and reliable control; safe to environment, humans, and animals; compatible with other rhizobacteria; and tolerance to desiccation, heat, oxidizing agents, and UV radiations (Nakkeeran et al. 2005).

To be an ideal formulation, it should have increased shelf life. It should not be phytotoxic to plants. It should be dissolved well in water and should release the bacteria. It should be cost-effective and should give reliable control of plant diseases. It should be compatible with other agrochemicals, and the carriers (for carrying the microbe) must be cheap and readily available for formulation development (Nakkeeran et al. 2005).

Types of Formulations

The formulations are in two forms, dry powder (solid) and liquid suspensions, which can be prepared in several ways.

Dry Powders

Organisms can be formulated into concentrated dry or wet powder for easy storage, transport, and application, which comprises of dust, granules, and briquettes for the preparation of formulations (Jones and Burges 1998). The types of powder preparation are wettable dry powder and moist powder culture. The wettable dry powder is normally spray-dried or lyophilized biomass with practically no free moisture for the growth of bacteria. In moist powder, culture organisms that do not form a mat of biomass can easily be formulated into a moist powder suitable for spraying, seed coating, or direct discharge into the soil (Paau 1998).

Different carrier materials used are peat, turf, talc, lignite, kaolinite, pyrophyllite, zeolite, montmorillonite, alginate, press mud, sawdust, and vermiculite (Kloepper and Schroth 1981; Vidhyasekaran and Muthamilan 1995; Rabindran and Vidhyasekaran 1996; Nakkeeran et al. 2005). Carriers increase the survival rate and shelf life of bacteria by protecting them from desiccation and also provide suitable microenvironment for rapid growth after their release. Rabindran and Vidhyasekaran (1996) used various formulations

Table 18.2 Partial list of commercially available formulations of PGPR as biocontrol agents as practiced in various parts of the world (Adopted from Sayyed et al. 2012)

Trade name	Biocontrol organism	Target pathogen/disease	Crop under application	Formulation	Mode of application
Bio-save 10[5]	*P. syringae*	*Botrytis cinerea*, *Mucor piriformis*, *Geotrichum*, and *Penicillium* sp.	Citrus and pome fruit	Wettable powder	Postharvest Drench, dip, and spray
BlightBan A506[6]	*P. fluorescence*	*Erwinia amylovora*	Almond, cherry, apple, potato, and tomato	Wettable powder	Postharvest Drench, dip, and spray
Conquer[8]¼Victus[26]	*Pseudomonas* sp.	*Pseudomonas tolaasii*	Mushroom	Aqueous biomass Suspension	Spray
Epic[11]	*Bacillus subtilis*	Various fungi	Cotton and legumes	Dry powder 5.5×10^{10} spores/g	Drip and potting mix in row
Mycostop[15]	*Streptomycetes* sp.	*Fusarium*, *Alternaria*, *Pythium*	Field ornamental and vegetable	Powder	Drench, spray, or through ligation
Intercept[14]	*P. cepacia*	*R. solani*, *Fusarium*, and *Pythium* sp.	*R. solani*, *Fusarium*, and *Pythium* sp.	–	–
Galltrol-A[13]	*A. radiobacter*	*A. tumefaciens* crown gall	Fruit, nut, and ornamental nursery plants	Bacterial suspension 1.2×10^{11} cfu	Suspension applied to seeds, seedlings, cuttings, root, stem, and as a soil drench
Kodiak[11], Kodiak HB, Kodiak AT	*B. subtilis*	*R. solani*, *Fusarium*, *Alternaria*, and *Aspergillus* sp.	Cotton and legumes	Dry powder 5.5×10^{10} spores/g	Slurry mixture for seed treatment

of *Pseudomonas* and found them effective in the management of sheath blight disease. Vidhyasekaran and Muthamilan (1995) prepared a formulation of *P. fluorescens* using various carriers such as talc, peat, lignite, vermiculate, and kaolinite to control the chickpea wilt. Kumar and Gupta (2010) studied the lignite-based formulation of *Azotobacter* to evaluate the bio-efficacy in wheat. Dry powder and dry granules are most successfully produced with spore-forming microbes, where drying processes such as spray-dry, freeze-dry, or air-dry are used for this purpose. The performance of microorganisms from the drying process varies with different microbes and with their physiological state. All dry formulations contain dormant microbes, whereas moist powder and granular formulations usually contain metabolically active microbes.

Liquid Suspensions

Solid-based formulations have some problems of survival. To overcome these problems, many formulations of the liquid-based inoculants have been introduced in recent years, which ensure longer survival, no contamination, ease of applicability, and tolerance to adverse environmental conditions. Liquid bioinoculants are special formulations containing not only the desired microorganisms and their nutrients but also the special cell protectants or substances that encourage longer shelf life and tolerance to adverse conditions (Vora et al. 2008). Liquid formulations typically are aqueous, oil-based, or polymer-based products (Jones and Burges 1998). Polysaccharides such as gums, carboxymethyl cellulose, and polyalcohol derivatives are frequently used to alter the fluid properties of liquid formulations (Paau 1988).

Materials used in liquid formulations are synthetic medium, sludge, baker's yeast, effluents, paneer-whey, and vegetable oil (Kremer and Peterson 1982; Hoben et al. 1991; Vendan and Thangaraju 2006; Pandey and Maheshawari 2007; Rebah et al. 2007; Albareda et al. 2008). In an experiment conducted with *Rhizobium* inoculants, liquid inoculant was found equally effective in comparison to the solid carrier-based inoculants (Gupta 2005). Vendan and Thangaraju (2007) developed cyst-based liquid formulation of *Azospirillum* using glycerol as a protective agent. Cyst-based bioinoculant formulations have twin advantages, i.e., longer shelf life and tolerance to adverse or harmful environmental conditions.

Liquid biofertilizer formulation could be considered as a potential strategy for improving the shelf life of biofertilizers. Unlike solid carrier-based biofertilizers, liquid formulation allows to add sufficient amount of nutrient, cell protectants, and inducers responsible for cell/spore/cyst formulation to ensure prolonged shelf life (Brar et al. 2012).

Mass Multiplication

The first requirement for the development of effective formulation is the production of PGPR strain in adequate amount. This requires the selection of proper, cheap, and easily available medium. In many cases, the production of bacterial strains is not easy because of the specific requirement of nutritional and environmental conditions. Mass production is achieved through liquid, semisolid, and solid state fermentation techniques.

The selected medium for mass multiplication should be inexpensive and readily available with appropriate nutrient balance. King's B broth or nutrient broth is used for the mass production of *Pseudomonas* and *Bacillus* species by using liquid fermentation technology (Manjula and Podile 2001; Nakkeeran et al. 2005). Slininger et al. (1996) reported that liquid culture formulation technology has to be optimized for the commercial exploitation of *P. fluorescens* intended for the management of take-all disease of wheat. Solid fermentation medium consisting of inert carriers with food bases was used for mass production of biocontrol agents (Lewis 1991). The solid fermentation technology is most suitable for fungal biocontrol agents.

Formulation Design

The success of formulation to suppress diseases or to enhance plant growth depends on the availability and shelf life of microbes because the shelf life of formulation decides the commercialization of bioinoculants. Formulation should support the viable nature of the product for the increased period of storage. Biocontrol product should have the minimum shelf life of 8–12 months for industrialization (Nakkeeran et al. 2005). Carrier material should not affect the viable nature of the biocontrol agent.

Formulation design also involves the type of formulation in which the choice of carrier is an important task. Many workers have evaluated the superiority of liquid formulations over the solid carrier-based formulations. Singleton et al. (2002) developed liquid formulation of *Rhizobium* by adding various additives in the yeast extract mannitol medium and claimed cell numbers of 1×10^{10} cells/ml in the liquid inoculant. Vendan and Thangaraju (2006) have developed liquid formulation of *Azospirillum* by using various cell protectants such as trehalose, polyvinylpyrrolidone, and glycerol, which resulted in increased shelf life by maintaining population level at 10^8 CFU/ml. Pandey and Maheshwari (2007) evaluated different carriers for the formulation of *Burkholderia* species strain MSSP and found that paneer-whey and wheat bran were efficient carriers in maintaining higher population.

Obstacles in Formulation Development

Formulation is the product of living organism so care needs to be taken to maintain the formulation at all the steps of production and maintenance. Production technology of bioformulations requires proper care and aid of sophisticated equipments to ensure availability of quality products in the market (Arora et al. 2010). During transport and distribution maintaining the temperature of the formulation is an important task. This, in turn, increases the cost of product. The earliest planning in the investigation of bioinoculants must anticipate the ultimate objectives and must meet commercial and scientific requirements (Burges and Jones 1998).

Shelf life of formulation is another obstacle because the storage of bioformulations requires special facilities and skills, which most producers, shopkeepers, and farmers do not possess. Shelf life is a culmination of several factors such as production technology, carrier and packaging material used, and mode and distance of transport. All these are desired to sustain the shelf life (Arora et al. 2010). Shelf life of formulation also depends on the performance of strain in field and laboratory condition. To avoid this problem of reduced survival rate of strain, the preparation should be air-dried and lyophilized (Kosanke et al. 1992, Fravel et al. 1998). Bacteria in the formulation remain inactive, resistant to environmental stresses, and insensitive to contamination and are more compatible with fertilizer application when the water content of the formulation is low (Bashan 1998).

Inconsistent field performance is the major obstacle associated with marketing of formulations. Many PGPRs work well in laboratory conditions, but they are unable to survive in fields, and therefore, this is an obstacle in the industrialization of formulation. These failures have raised concerns about the perspective of the great practical potential offered by microbial releases into soils. A key factor involved in the failure is the rapid decline of the size of the population of active cells. The active cells decline to a level at which the formulation becomes ineffective when introduced into the soil and the desired objective is not achieved. As soil is a heterogeneous system with a mixed biota, the formulation added in soil is subjected to rhizosphere competence, interaction with already adopted soil microflora, and survival in new habitat. A number of factors such as soil composition, temperature, relative humidity, composition of root exudates, presence of recombinant plasmids, as well as the interactions with other soil biota can affect the persistence of a PGPR on the root system making it difficult to predict the behavior of the bacterial strain under natural conditions. Therefore, PGPRs that are effective in the laboratory may not show any significant impact on plants under field conditions (Glick et al. 1999).

Commercial Aspects

With increasing population and public interest, the demand of organically grown food is also increasing. To fulfill the requirement of food, the annual requirement and present production capacity for different biofertilizers not only show a tremendous gap but also indicate the potential for organized production. The requirement for *Rhizobium* cultures to cover the entire cultivation area in India will be around 15,000 tons of carrier-based material, of which the present production is only around 800 tons. Similarly, 400,000 tons of BCA are required to cover the entire rice cultivation area in India. This indicates the need to augment efforts and to plan a phased strategy (Tilak and Saxena 1986).

An ongoing attempt to promote biofertilizer in agriculture through public intervention is expected to increase the commercial market of biofertilizers. To attain production targets of biofertilizers, their production, distribution, and promotion are some of the aspects that need to be focused.

In the recent past, large amount of research has been carried out on the PGPR, which has also led to the development of commercial products. Representative commercially available bacterial biocontrol products are listed in Table 18.2. It has been observed that biocontrol products used to date control possess relatively narrow spectrum of diseases control on a particular host crop. Hence, before approaching for commercial production, a company must asses many factors including demand for the product, potential market size, and existing competing formulation of the bioproducts.

Conclusion

The complex environment of the rhizosphere and its community structure is not fully understood. Hence, we can expect to see new PGPR products becoming available from new emerging strains of PGPR. The success of these products depends on our ability to understand the mechanism of action of PGPR and manage the rhizosphere to enhance survival and competitiveness of these beneficial microorganisms.

In the past few years, there has been an increasing interest in utilizing PGPRs as adjuncts to agricultural practice and environmental cleanup. Moreover, with this interest there have been major efforts worldwide to better understand many of the fundamental mechanisms that PGPRs use to facilitate plant growth and biocontrol. An effective PGPR could be developed for disease control only after understanding its performance under natural environmental conditions and in the presence of other competing microbiota in which it is expected to perform large amount of research has been devoted to PGPR and BCAs (Nielsen et al. 2000; Bloemberg and Lugtenberg 2001; Pieterse et al. 2001; Thrane et al. 2000; Sayyed and Chincholkar 2009; Sayyed and Patel 2011; Sayyed et al. 2012, 2013). In nature, agriculture crops are exposed to diverse environmental conditions and gambling of monsoons, which alter the microclimatic conditions existing around the infection court. A thorough knowledge on the mechanisms and performance related to disease control will help in the selection of promising candidates that suit industries to produce reliable commercial products (Collins and Jacobsen 2003).

Future Prospective

Rhizosphere management will require consideration of soil and crop cultural practices as well as inoculant formulation and delivery. Biotechnology can be applied to further improve strains that have appreciated qualities, namely, formulation ease, stability, and competent root colonization, by creating transgenic strains that combine multiple mechanisms of action. Genetic manipulation of PGPR strains to enhance colonization and effectiveness may involve addition of one or more traits associated with plant growth promotion. The use of PGPR with known functions is of interest as these formulations may increase consistency in the field. They offer the potential to address multiple modes of action, multiple pathogens, and temporal or spatial variability. PGPRs offer an environmentally sustainable approach to increase crop production.

There is no doubt that PGPRs are the potential tools for sustainable agriculture not only in India but also all over the world. The use of biofertilizers (PGPRs) in preference to chemical fertilizers is always welcome taking into consideration its suitability for agriculture. Biofertilizers are always beneficial in terms of soil fertility, ecological health, etc. As the use of biofertilizers, till so far, is grossly inadequate in India, more emphasis needs to be given on their production, consumption, and proper distribution. Selecting the strains with multiple traits for biocontrol will be effective in the field of PGPR.

Designing an effective formulation with the increased shelf life and tolerance to adverse condition is always a challenging task, but the liquid formulation has given an option to this. This has enhanced the shelf life, but has also increased the cost of production. To reduce the production cost of formulation, cheap raw materials need to be used or the shelf life of solid formulations needs to be improved as the commercialization depends on the shelf life of formulation.

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Effects of Soil Environment on Field Efficacy of Microbial Inoculants

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Ekta Khare and Naveen K. Arora

Abstract

Although many microorganisms show good performance in specific trials, this is often not translated into consistent, effective plant growth promotion and biocontrol in diverse field situations. The key factors involved in the lack of success are rapid decline in the size of populations of active cells to levels ineffective to achieve the objective and variable production of required metabolites or poor colonization, following the introduction into soil. The physical, chemical, and physicochemical nature of soil and its indigenous microorganisms and predators influence the microbial population both quantitatively and qualitatively. Soil abiotic factors (e.g., texture, pH, temperature, and moisture) exert their (direct) effect on inoculant population dynamics by imposing stresses. On the other side, trophic competitions and antagonistic/synergic and predatory interactions with the resident microbial and fauna populations determine the field efficiency of inoculants. The aim of this review is to throw light on different soil environmental conditions that affect the survival of inoculated microbial strains in the field. A proper characterization of target soils and rhizospheres as habitats for introduced microbes is a key to the development of bioformulations that support beneficial microorganisms in the soil.

Introduction

The increased use of chemical pesticides and fertilizers in agriculture helped the world in achieving self sufficiency in food grain production. However, it has also polluted the environment and caused slow deterioration of soil health. The chemical residues in the food products are also causing harm to human beings and other living organisms. To combat these problems and in

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the light of sustainable agriculture, plant growth-promoting microorganisms (PGPMs) as green technology are now being greatly used (Sumathi et al. 2012). Plant growth benefits may be attributed mainly to three mechanisms: (1) PGPMs acting as biofertilizers (such as nitrogen-fixing bacteria and phosphate-solubilizing bacteria) assist plant nutrient uptake by providing fixed nitrogen or other nutrients; (2) phytostimulators (microbes-expressing phytohormones such as *Azospirillum*) can directly promote the growth of plants, usually by producing plant hormones; and (3) biological control agents (such as *Trichoderma*, *Pseudomonas*, and *Bacillus* spp.) protect plants against phytopathogenic organisms (Compant et al. 2005; Trabelsi and Mhamdi 2013). Although PGPMs occur in soil, usually their number is not high enough to compete with other indigenous microorganisms commonly established in the rhizosphere. Therefore, for agronomic utility, inoculation of plants with target microorganisms at a much higher concentration than those normally found in soil is necessary to take advantage of their beneficial properties for plant yield enhancement (Subba Rao 1993).

Formulation/inoculant is an industrial art of converting a promising laboratory-proven microbe to a commercial field product (Bashan 1998). Many bacterial inoculants exert their beneficial effects in laboratory culture, a lower number are successful in a laboratory greenhouse, and a much lower number are functioning under practical conditions, i.e., in a commercial greenhouse or in the field (Lugtenberg and Kamilova 2009). The soil being a heterogeneous, unpredictable environment, the inoculated microbe finds it often difficult to establish a niche for survival among the competitors and predators. Uncertainty about the product performance can lead to poor demand from end users, the farmers. Understanding the reasons for the failures in the field may lead to the development of bioformulations that are effective in natural conditions.

> "…the best type go all over the world, fitting in so perfectly with their background that not even the inhabitants notice they are strangers; in other words they achieve the highest accomplishment possible" (Post 1922)

The "highest accomplishment" for biocontrol and PGP bioformulation lies in "fitting in" to the host environment and being able to tolerate, manipulate, or evade plant defense responses. As climatic conditions, soils, plants, and microorganisms are all variable and/or diverse, there is no general rule for how introductions into soils can be optimized. However, it is clear that since soil generally represents a hostile environment to microbial introductions and since microbial cells in soil are subjected to a range of adverse abiotic and biotic conditions (Fig. 19.1), the success of the application of microbes depends to a large extent on how favorable to its survival and functioning the target environment is or can be made, in terms of either natural or induced ecological selectivity or available protective niches. Abiotic soil factors (e.g., texture, pH, temperature, and moisture) exert their (direct) effect on inoculant population dynamics by imposing stresses of various natures on the cells (van Elsas et al. 1991; Evans et al. 1993). They can also act indirectly by affecting the activity of the indigenous soil microflora. Both abiotic and biotic factors regulate the production of antifungal metabolites under in vitro and in situ conditions (Dwivedi and Johri 2003; Khare et al. 2011). The dynamics of the inoculant population, i.e., the interactions of the inoculants with and response to their environment (predation, antagonism, and death/growth), is responsible for inconsistent performance at the soil microhabitat level (van Veen et al. 1997). Since effectiveness of bioformulation and soil properties is interrelated with one another, the understanding of and recognizing soil properties are important for making sound decisions regarding the use of microbial bioformulations. This review presents a general discussion on the factors that cause the decrease in numbers and activity of PGPMs following their introduction into soil (Fig. 19.2).

Effect of Soil Abiotic Factors

Soil Texture

Soils are made up of four components: minerals, air, water, and organic matter (Brady and Weil 2002). In most soils, minerals represent around

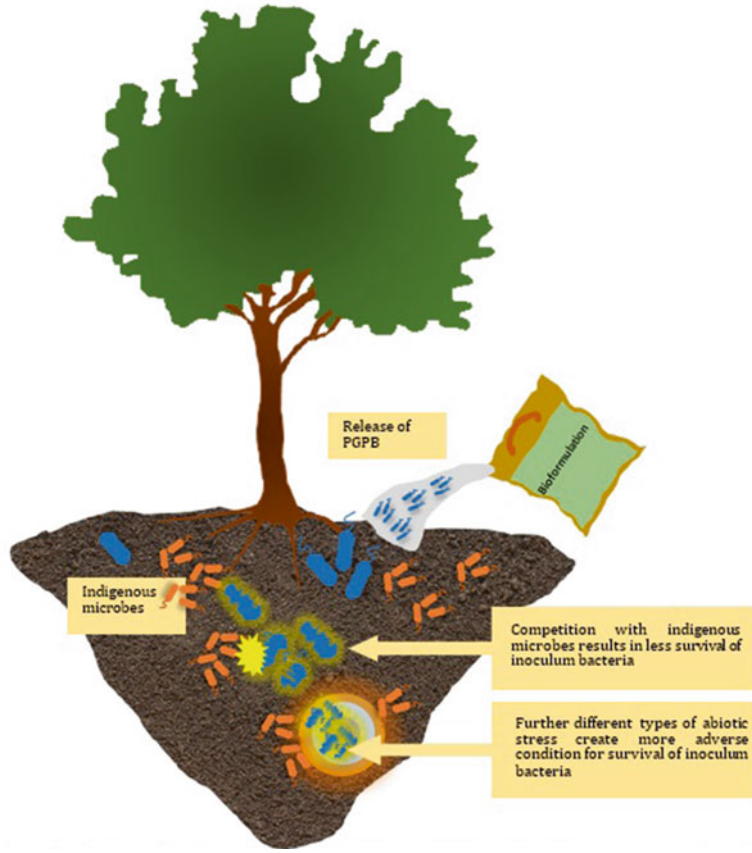
Release of PGPB
Bioformulation
Indigenous microbes
Competition with indigenous microbes results in less survival of inoculum bacteria
Further different types of abiotic stress create more adverse condition for survival of inoculum bacteria

Fig. 19.1 General state of inoculated cells in the hostile rhizosphere ecosystem

45 % of the total volume, water and air about 25 % each, and organic matter from 2 to 5 %. The mineral portion consists of three distinct particle sizes classified as sand, silt, or clay. Sand is the largest size particles that can be considered soil. Sand is largely the mineral quartz, though other minerals are also present. Silt particles are much smaller than sand, but, like sand, silt is mostly quartz. The smallest of all the soil particles is clay. Mineral surfaces in soils are mainly provided by the very small particles (<2 μm) that constitute the soil clay fraction. This fraction is a mixture of phyllosilicates, oxides, and hydroxides and, in some soil types, short-range-order minerals like allophane and imogolite. Traditional concepts were highly focused on the role of phyllosilicates as dominating mineral surfaces for the formation of organo-mineral associations (Theng 1979; Stevenson 1982). Clays are quite different from sand or silt and contain appreciable amounts of plant nutrients (Silver et al. 2000). Clay has large surface area resulting from the platelike shape of the individual particles. The textural designation of a soil is derived from the relative portions of sand, silt, and clay. Another soil characteristic – soil structure – is different from soil texture. Structure refers to the combination or "aggregation" of sand, silt, and clay particles into larger secondary clusters. If you grab a handful of soil, good structure is apparent when the sand, silt, and clay particles are aggregated into granules or crumbs (Nimmo and Perkins 2002).

Both texture and structure determine pore space for air and water circulation, erosion resistance, looseness, ease of tillage, and root penetration (Rieu and Sposito 1991). However, while texture is an innate property of the native soil and does not change with agricultural activities,

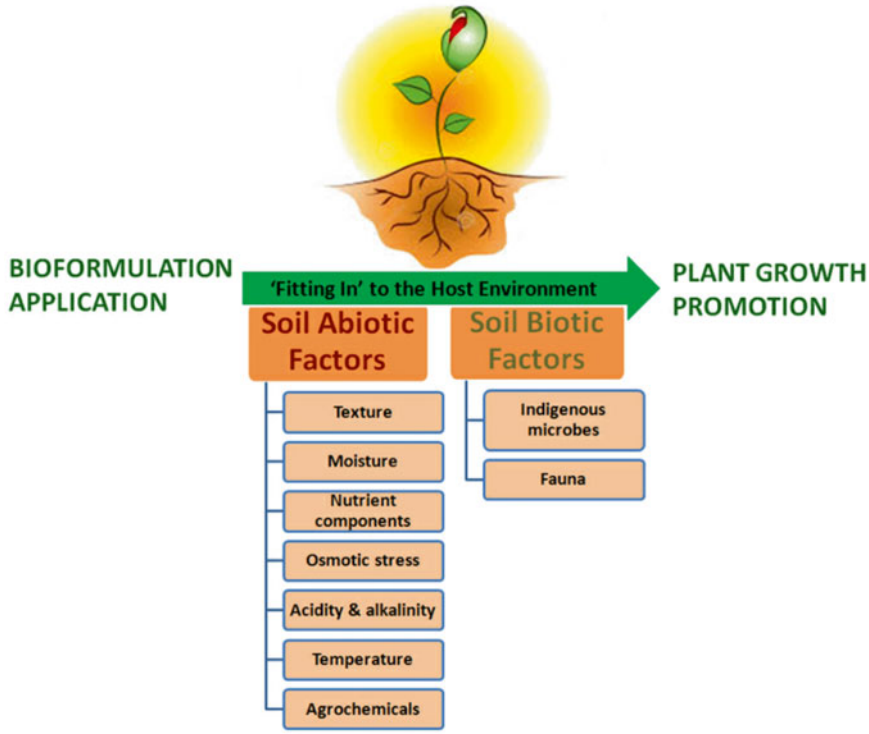

Fig. 19.2 Soil environmental conditions that are necessary to evaluate, for the development of bioformulation to achieve the highest accomplishment possible

structure can be improved or destroyed readily through our choice and timing of farm practices. A well-aggregated soil has increased water entry into the soil, increased aeration, and increased water-holding capacity (Rieu and Sposito 1991). An aggregate formed by physical forces (wetting and drying, freezing, and thawing) becomes stabilized (will remain intact when wet) through microbial processes involving organic matter decomposition and its by-products – chiefly gums, waxes, and other glue-like substances. These by-products cement the soil particles together forming water-stable aggregates. Aggregated soils also prevent glazing or crusting of the soil surface (Boyle et al. 1989). Previous studies have shown that soil types influence the structure of microbial communities, especially bacterial population among soils of different textures (Garbeva et al. 2004; Fang et al. 2005). On the other hand, no significant differences were noticed among average counts of soil fungi due to soil textures (Hamarashid et al. 2010).

The soils in good physical condition have better aeration and moisture content which is essential for optimum microbial activity. Soils of different texture differ in particle size composition and, thus, in pore size distribution. The pores between particles of solid material are just as important to the nature of the soil as are the solids themselves (Miller and Donahue 1995), since it is in the pore space that air and water circulate, roots grow, and most of the microbial population live. A structure of good quality implies a good quality of pore space, with good continuity and stability of pores and a good distribution of the pore size, including both macro- and micropores (Cabeda 1984). Moisture is held in the micropores. Water moves in the macropores, and these tend to be occupied by the air constituting the soil atmosphere. Hence, pore size distribution strongly determines the fate of introduced microorganisms, and differences in the behavior of bacteria released into different textured soils may be related to differences in the protective pore

spaces present in these soils. The gaps between sand particles tend to be large favoring aeration but also result in rapid water loss. In contrast, pores between clay particles tend to be small, favoring water retention (Ashman and Puri 2002) but decreased aeration (Miller and Donahue 1995). In this way, soil texture influences most soil processes. In a simplified explanation, it not only determines its moisture and aeration characteristics (Brady and Weil 2002) but ultimately its ability to store and supply organic matter for plant and microbial use. For instance, clays due to their large sorption area as well as their electrostatic nature have a greater tendency to adsorb and retain organic matter into stable aggregates (Ashman and Puri 2002). Colonization of soil particles and aggregates is assumed to be vital to ultimate inoculant survival in soil (Hattori and Hattori 1976).

van Veen et al. (1997) reported higher survival levels of inoculants in fine-textured (clay) than in coarse (sandy) soils. The effects of, for instance, clay surfaces in conferring protection upon bacteria have been convincingly documented by Marshall (1975). These bacterial populations are top-down controlled by the grazing of bacterivorous nematodes and protozoa (Ingham et al. 1986; Moore et al. 1988; Wardle 2002). Nematodes are ubiquitous residents of soils and sediments in most ecosystems. Some soil nematode species feed on bacteria or fungi, while others are omnivorous, predaceous, or parasites of plant roots (Yeates et al. 1993). Soil nematodes range in size from 0.3 to 3 mm and are active in water-filled pores in the soil matrix and in water films that coat soil particles. Due to their small size and inability to migrate over long distances, soil nematodes are closely dependent on the immediate environmental conditions in soil pores to feed and reproduce (Treonis and Wall 2005). Bacterivorous soil protozoa accumulate in "hot spots" of microbial activity as in the humus region or closed to plant roots, attracted by their prey (Griffiths 1990; Hausmann and Hülsmann 1996; Scheu et al. 2005) reaching densities of 10^4–10^7 active protist individuals per gram dry weight forest soil (Adl et al. 2006). Bacterivorous protozoa are more important than nematodes since they have access to bacteria living in small pore spaces unavailable to nematodes (Bamforth 1997). Each microbial species has different optimal conditions, but overall bacterial activity is highest when pore spaces are about 60 % water filled. Soil texture and porosity determine the amount of space available for soil organisms and for the movement of air and water through the soil. Thus, porosity, aeration, and moisture levels are linked (Chapin et al. 2002).

Soil Moisture

The survival and activity of microorganisms may depend on their distribution among microhabitats and changes in soil moisture (Orchard and Cook 1983; Sylvia et al. 2005). It is one of the important factors influencing the microbial population and their activity in soil. In soil, water is held as films on particle surfaces and within soil pores (Stengel and Gelin 2003) through hydrogen bonding (Miller and Donahue 1995). Therefore, the higher the clay or organic matter contents of a soil, the larger the amount of water it can store, but also more tightly it is bond to the large surface area (Brady and Weil 2002). However, if the water is held too tightly, it becomes unavailable to plants and microorganisms, as they can only extract water at certain tensions. "Tension" here refers to the measure of suction required in order to extract water from the soil (Miller and Donahue 1995; Ashman and Puri 2002). Perhaps the most important abiotic factor affecting bacterial dispersion in soil is water (Madsen and Alexander 1982). Understanding bacterial movement in soil is also important for manipulating colonization of plant roots and for optimizing the dispersal of inoculants used in biocontrol strategies (Turnbull et al. 2001). The inability of the introduced organisms to move from the site of inoculation (usually the seed) and establish population in the rhizosphere and on plant roots is one of the main problems (Suslow 1982). The ability to colonize distal parts of the rhizosphere from an initial point of inoculum, especially in the absence of percolating water flow, has been termed "mobility" (Misaghi et al. 1992) and has been

implicated as an important trait for biocontrol in the rhizosphere. Preferential water flow has been shown to move along the rhizoplane surface in soil systems (Natsch et al. 1996), and bacteria can be carried in large numbers along root surfaces by this mechanism (Parke et al. 1986; Kluepfel 1993). Chao et al. (1986) demonstrated that the depth of rhizosphere colonization by bacteria was influenced by the volume of percolating water added to a root system. In field condition, bacteria inoculated on seed would also be distributed along roots by preferential flow from rainfall (Natsch et al. 1996), although this movement would also be influenced by other environmental factors.

Since the possibilities for directed movement of bacteria in soil are limited (Foster 1988), microbial cells upon introduction into soil are probably not immediately localized in protective soil sites. Specific action may be needed to localize as many inoculant bacteria as possible at protective sites. Postma et al. (1989) showed that manipulation of the introduction method affected the localization of inoculant bacteria in protective microhabitats in soil. In their experiments, recipient soil was dried to different moisture levels prior to addition of bacterial cells. The bacterial cells would reach the soil pore space with the water of the inoculum. In the case of moist soil at the moment of introduction, the water present in small, potentially protective pores is assumed to act as a physical barrier to the entry of cells, resulting in the localization of the inoculum in the relatively large pores. In initially dry soil, a greater proportion of smaller pores are not water filled and hence could be reached by the inoculant cells with the incoming water. This implies that bacterial cells introduced into initially wet soil would be accessible for predation to a larger extent than would cells introduced into initially dry soil (van Veen et al. 1997).

Water (soil moisture) is useful to the microorganisms in two ways, i.e., it serves as source of nutrients by supplying hydrogen/oxygen to the organisms and as solvent and carrier of other food nutrients to the microorganisms. Microbial activity and population proliferate best in the moisture range of 20–60 %, typically due to the increased diffusivity of substrates in soil solution and the activation of dormant microorganisms by available water. Under excess moisture conditions/water-logged conditions, due to lack of soil aeration (oxygen), anaerobic microflora become active and the aerobes get suppressed. While in the absence of adequate moisture in soil, some of microbes die out due to tissue dehydration, and some of them change their forms into resting stages/spores or cysts to tide over adverse conditions (Davidson 1993). One of the immediate responses of rhizobia to water stress (low water potential) concerns the morphological changes. Rhizobia from *Prosopis* (mesquite) (Shoushtari and Pepper 1985) and *Rhizobium meliloti* (Busse and Bottomley 1989) showed irregular morphology at low water potential. The modification of rhizobial cells by water stress will eventually lead to a reduction in infection and nodulation of legumes (Hunt et al. 1981). A reduction in the soil moisture from 5.5 to 3.5 % significantly decreased the number of infection threads formed inside root hairs and completely inhibited the nodulation of *Trifolium subterraneum* (Worrall and Roughley 1976). The decrease in water availability may cause a change in the physiology of microbes as they adjust to more desiccating conditions (Stark and Firestone 1995). For example, one microbial response to water stress is the accumulation of intracellular solutes for osmoregulation (Harris 1981). Microbial growth may be hindered biochemically by a high intracellular concentration of solutes, or growth efficiency may be lowered because of costs of osmoregulation (Harris 1981), because the efficiency with which microbes use carbon substrates to create biomass is directly related to the tendency to immobilize other mineral nutrients into biomass (Herron et al. 2009).

Oxygen limitation and reducing conditions were likely to be present in soil located immediately above the plow plan, where decomposing crop residues were apparent and the soil was water logged. Mascher et al. (2000) reported that abiotic stress resulting from a combination of oxygen limitation and reducing conditions had a role in the formation of viable but nonculturable (VBNC) cells of inoculated *Pseudomonas*

fluorescence in field experiment. The exposure of *P. fluorescens* to a combination of these two stress factors or to a high intensity of a single type of stress in vitro resulted in mixed populations of culturable and nonculturable cells, and VBNC cells represented a significant proportion of the latter. Once in soil, the VBNC cells thus obtained did not display enhanced persistence compared with culturable cells, suggesting that their physiological state did not represent a successful adaptive response to adverse environmental conditions. Therefore, optimum soil moisture (range 20–60 %) must be there for better population and activity of microbes in soil.

Nutrient Components

In general, population sizes of PGPMs decline more or less rapidly following introduction into a natural soil, and growth of introduced populations in microbiologically undisturbed soils is a rare phenomenon. One of the main reasons is the scarcity of available nutrient sources to microbes in soil (van Veen et al. 1997). Soil concentrates C, N, P, Fe, essential metals, and various organic compounds above surrounding environmental levels. These essential nutrients may be acquired from soil mineral surfaces (Vaughan et al. 2002). Many important microbial processes can be influenced by minerals, including energy generation, nutrient acquisition, cell adhesion, and biofilm formation (Hochella 2002; Brown et al. 2008). The amount of organic matter that is stabilized in soils can be predicted from the total mineral surface area (Hassink 1997; Six et al. 2002). Soil organic matter (SOM) plays very significant roles in soil. According to a review by Bloem et al. (2006), SOM is usually considered to comprise two main pools: the "living" (e.g., roots and microbial populations) and the "nonliving" (nutrients of different ages and origins) pool. For the purpose of this review, microorganisms will be treated independently of SOM for simplification. SOM strengthens soil aggregates, increases water retention, buffers soil pH, chelates metals, interacts with xenobiotics, and retains cations and anions in soil systems. In doing so, SOM largely determines the physical, chemical, and biological qualities of a soil (Stevenson 1994; Etebu and Osborn 2012). The organic matter in soil being the chief source of energy and food for most of the soil organisms has great influence on the microbial population. Wididana (1990) conducted an experiment in which a 0.1 % aqueous solution of molasses was applied to soil. It resulted in a significant increase in the number of indigenous microorganisms in soil over that of the unamended control. Nakkeeran et al. (2005) mentioned that the performance of bioformulations can be increased by the incorporation of enrichment materials.

Microorganisms need nutrients from the environment for cell growth and division. Even though carbon is essential for microbial growth and survival, relatively few studies have examined the effects that carbon substrates have on the expression of microbial genes in a soil environment. Studies with *lac*Z as a reporter gene controlled by a carbon starvation-induced promoter in *P. fluorescens* in natural soil revealed that *lac*Z gene expression increased shortly after introduction of the cells into soil (van Overbeek et al. 2006). Apparently, the introduced cells indeed sensed carbon-limited conditions in soil, and this resulted in a physiological response which involved the induction of the carbon starvation-inducible reporter gene.

Nitrogen is an essential macronutrient required by microorganisms to support cell growth. Since nitrogen concentrations and form vary in soil environments, microbial formulations applied in the soil must be able to adapt in order to continue to grow and divide. In response to a decrease in cellular ammonia and consequently glutamine, transcription in the gene *gln*N encoding for the enzyme glutamine synthase increases. The increase in this enzyme allows for an enhanced ability to use small amounts of available ammonia. Concurrent with the transcriptional activation of *gln*N, other nitrogen regulatory genes are activated causing the formation of many enzymes and permeases responsible for utilizing other N sources (Magasanik 1996). Enzymatic activity studies coupled with gene expression determinations have also shown that nitrogen fixation is induced when other sources of nitrogen are not

available (Bürgmann et al. 2003). Nitrogen fixation was induced through the provision of excess carbon source, causing nitrogen to be a limiting nutrient. This corresponded with the study of Jensen et al. (1998), where addition of excess carbon and phosphorus resulted in a 1,000-fold increase of bioluminescence, as measured by a nitrogen-starvation-inducible gene.

Phosphorus (P) in soil is subject to extensive physicochemical and biological reactions with only a small component of total soil P being in a form directly available for plant or microbial uptake. Nutritional constraints linked to scarcity of available P can shape soil microbial community composition in highly weathered soils (Cruz et al. 2009). It is known that every aspect of the process of formation of the N_2-fixing nodule is limited by the availability of P. Legumes like alfalfa, clover, common bean, cow pea, and pigeon pea show a high positive response to P supplementation (Gyaneshwar et al. 2002). In *Rhizobium*, P limitation can result in higher P transport rates and the induction of alkaline phosphatase (Al-Niemi et al. 1997). Moreover, while microorganisms may have capacity to directly solubilize P to meet their own requirements, subsequent benefits to plants may only occur following turnover of the microbial biomass, i.e., through mineralization as the bacteria die (Gyaneshwar et al. 2002). There is also a need to demonstrate that microorganisms in the rhizosphere have the ability to deplete inorganic forms of soil P over and above that by plants alone. There are also reports that inorganic phosphate represses antibiotic production by rhizobacteria posing a major concern about potential adverse effects of phosphate fertilizers commonly used in agriculture on not only introduced biocontrol agents but also on indigenous populations of antagonists (Slininger and Jackson 1992; Millner et al. 1996; Dutta and Podile 2010).

Iron is the fourth most abundant element on earth (Ma 2005); however, in aerobic soils, iron is mostly precipitated as hydroxides, oxyhydroxides, and oxides so that the amount of iron available for assimilation by living organisms is very low, ranging from 10^{-7} to 10^{-23} M at pH 3.5 and 8.5, respectively. Both microbes and plants have a quite high iron requirement (i.e., 10^{-5}–10^{-7} and 10^{-4}–10^{-9} M, respectively), and this condition is more accentuated in the rhizosphere where plant, bacteria, and fungi compete for iron (Guerinot and Ying 1994). Cellular iron deficiency in some bacteria induces the synthesis of low-molecular weight siderophores, molecules with an extraordinarily high affinity for Fe^{+3} (Ka ranging from 10^{23} to 10^{52}), as well as membrane receptors able to bind the Fe–siderophore complex, thereby allowing iron uptake by microorganisms (Neilands 1981). Plant iron nutrition can affect the structure of microbial communities in the rhizosphere. For example, transgenic tobacco that overexpresses ferritin and accumulates more iron than non-transformed tobacco has less bioavailable iron in the rhizosphere. As a consequence, the composition of the rhizosphere microbial community differed significantly when compared to non-transformed tobacco lines (Robin et al. 2006).

Duffy and Défago (1999) studied the environmental factors that modulate the biosynthesis of antibiotics and siderophores by the disease-suppressive strain *P. fluorescens* CHAO. The production of the antibiotic 2,4-diacetylphloroglucinol (DAPG) was stimulated by Zn^{2+}, NH_4Mo^{2+}, and glucose, and production of pyoluteorin was enhanced by Zn^{2+}, Co^{2+}, and glycerol and was repressed by glucose. The production of the siderophore pyochelin was increased by Co^{2+}, fructose, mannitol, and glucose. Comparison of strain CHAO with a genetically diverse collection of 41 *P. fluorescens* biocontrol strains indicated that the effect of some factors like the stimulation of DAPG by Zn^{2+} and glucose was strain dependent (Duffy and Défago 1999). Fe^{3+} and sucrose have been reported to increase the levels of DAPG in *P. fluorescens* F113, whereas in *P. fluorescens* Pf-5 and CHAO, DAPG was stimulated by glucose (Duffy and Défago 1999; Nowak-Thompsan et al. 1994). In *P. fluorescens* strain S272, the highest DAPG yield was obtained with ethanol as the sole source of carbon (Dwivedi and Johri 2003). Microelements, such as Zn^{2+}, Cu^{2+}, and Mo^{2+}, have been found to stimulate DAPG production in *P. fluorescens* CHAO (Notz et al. 2001). Fructose, mannitol, and a mixture of

Zn^{2+} and NH_4Mo^{2+} increased pyrrolnitrin production (Fernando et al. 2005). Apart from being an excellent nutrient supply to plants and microorganisms, organic matter makes the soil less compact, therefore providing better aeration and water movement, as well as protecting it from erosion. This is particularly important in hot and dry environments, whereby organic covering (mulch) on the soil prevents, rapid moisture loss, and excessive temperature increases are prevented (Miller and Donahue 1995).

Osmotic Stress

High concentrations of salt in soil can have a detrimental impact on its physical, chemical, and biological properties (Rengasamy 2006) and unbalance the ecosystem. Salts in soil cause osmotic stress to plants and soil biota by decreasing the osmotic potential of the soil solution. Fungi are more sensitive to salinity than bacteria (Pankhurst et al. 2001; Wichern et al. 2006). The study by Sardinha et al. (2003) found decreasing ergosterol concentration with increasing salinity which indicates fungal abundance decreased with salinity. Salinity can stress or even kill soil microorganisms which drive nutrient cycling (Wichern et al. 2006). Key ecological functions such as nitrogen transformation are reduced or delayed if a large proportion of a microbial population dies (Schimel et al. 2007). Salinity can also cause nutrient imbalance as sodium and chloride may compete with nutrient cations and anions in uptake and affect key metabolic functions. The reductions in soil microbial activity in saline soil result in poor plant growth, which reduces crop yields and organic matter input into the soil (Summer et al. 1998); these lead to environmental problems such as erosion and eutrophication (Rengasamy 2006). Mavi and Marschner (2013) reported that in the short term, addition of a readily available and easily decomposable source of energy improves the ability of microbes to tolerate salinity. The results also suggested that in saline soils, irrespective of the C substrate, N addition has no impact, or a negative impact, on microbial activity and growth.

Soil salinity and related problems generally occur in arid or semiarid climates where rainfall is insufficient to leach soluble salt from the soil or where surface or internal soil drainage is restricted. Another cause of increase in soil salinity is the application of fertilizers, for example, the use of potassium as fertilizer, which can form sylvite, a naturally occurring salt (Romheld and Kirkby 2010). The osmotic potential ($\Psi\pi$) which is the result of interactions of salts with soil water is important in saline soils (Papendick and Campbell 1981). Moreover, the reduction in soil water content by evapotranspiration that concentrates the salts in the remaining solution leads to the changes in osmotic potential of the soil solution. Water molecules are attracted to electrostatically charged cations such as hydrogen, sodium, potassium, and calcium. Therefore, increasing concentrations of salt in soil solution restrict the availability of the soil water to microorganisms and plant roots. In extreme cases, water moves from the microbial cells to the lower osmotic potential of the soil solution (Brady and Weil 1999). The effect is always more pronounced in the rhizosphere because of the increase in water absorption by the plants due to transpiration (Jiang et al. 2007).

To minimize the effect of low osmotic potential in saline soils, the tolerant microorganisms accumulate solutes in the cell to counteract the concentration gradient between cell cytoplasm and soil solution (Killham 1994). Polyhydroxyalkanoates (PHAs) are biopolymers synthesized by various bacteria as carbon, energy, and reducing power storage material in response to conditions of salinity stress (Arora et al. 2006; Khare et al. 2014). Synthesizing osmoregulatory compounds requires large amounts of energy: 30–110 ATP (adenosine triphosphate) compared to 30 ATP for cell wall synthesis (Oren 1999). Thus, high concentrations of salts can not only kill sensitive microorganisms but also reduce the activity of surviving organisms because of the metabolic burden imposed by the need for stress tolerance mechanisms (Schimel et al. 2007). Khare et al. (2011) reported reduction in production of plant growth-promoting and biocontrol metabolites with increase in osmotic stress. High

salinity reduces the available phosphorus by sorption processes and results in low solubility of Ca-P complexes (Chookietwattana and Maneewan 2012). The effect of salinity on microbial biomass and community structure can be variable. The varying effects may be due to the salinity level, soil type, and microbial community composition as well as water content. Selection of salt-tolerant strains from arid or semiarid habitats can serve the purpose of inoculants in such habitats. Kumar et al. (1999) reported that biological nitrogen fixation and fertility of saline soil can be increased by planting salt-tolerant tree legumes along with inoculation with salt-tolerant rhizobia. Highly salt-tolerant strains of rhizobia have been reported by various workers from saline and arid soils (Singleton et al. 1982; Abd Alla and Abdel Wahab 1995; Kumar et al. 1999).

Mascher et al. (2000) reported that high osmotic stress in soil results in mixed populations of culturable, nonculturable, and VBNC cells of inoculated pseudomonads. High NaCl concentrations do not have the same effect on all Gram-negative bacteria. The culturability of cells of *P. fluorescens* AH9 was reduced after incubation in 1.7 M NaCl but not after incubation in 1 M NaCl (Jørgensen et al. 1994). In contrast, *Pseudomonas aeruginosa* PAO1 (Velasco et al. 1995) and *Escherichia coli* (Roth et al. 1988) were already affected at 0.7 and 0.8 M NaCl, respectively. This may, in part, reflect differences in osmotic potential between the habitats from which these bacteria originate. Interestingly, tolerance to high NaCl concentrations was suggested to be an important bacterial property for successful colonization of the root (Loper et al. 1985; Miller and Wood 1996). Once introduced in soil, VBNC cells did not display enhanced persistence compared with culturable cells, which suggests that this VBNC state did not represent a physiological strategy to improve survival under adverse conditions. Successful commercial application of microbial inoculants to improve crop growth and yield in saline soil implies that the inoculants are also salt tolerant, which highlights the potential of using microorganisms from saline habitats. It is important to conduct screening experiments with different soil types under controlled conditions and the consideration of water potential instead of electrical conductivity only.

Soil Acidity and Alkalinity

Soil pH has numerous effects on physiological, morphological, and metabolic responses of soil microorganisms, as well as the availability of mineral nutrients (Fierer and Jackson 2006). Agricultural residues and other plant materials can have a liming effect when added to soil in the absence of plants and leaching (Tang et al. 1999; Tang and Yu 1999; Sakala et al. 2004; Xu et al. 2006b). Increases in pH after the addition of residues are purported to occur due to the decarboxylation of organic anions (Yan et al. 1996; Tang and Yu 1999). Other studies have suggested that basic cations which are released during decomposition increase the pH (Pocknee and Sumner 1997; Noble and Randall 1999). Nitrogen (N) cycling is considered a main mechanism for pH change, with the conversion of organic N to NH_4^+ consuming H^+ and subsequent conversion to NO_3-releasing $2H^+$ (Xu and Coventry 2003; Xu et al. 2006a). Leaching of nitrate results in net acidification and would otherwise be balanced by nitrate uptake by the plant. Furthermore, association/dissociation reactions of H^+ with residue surfaces, organic compounds, and the soil matrix may also occur and contribute to the pH change. Soil alkalinity (pH>7) presents itself in soils where the material is calcareous or dolomitic. Sodic soils occur where there has been an accumulation of exchangeable sodium, naturally or under irrigation. Such soils have high concentrations of OH^- ions associated with high contents of bicarbonates and carbonates.

Soil nutrient availability is greatly influenced by soil pH. With the exception of phosphorus, which is most available within a pH range of 6–7, macronutrients (N, K, Ca, Mg, and S) are more available within a pH range of 6.5–8, while the majority of micronutrients (B, Cu, Fe, Mn, Ni, and Zn) are more available within a pH range of 5–7. Outside of these optimal ranges, nutrients are available to microbes and plants at lesser

amounts (Haby 1993). With the exception of molybdenum (Mo), micronutrient availability decreases as soil pH values approach 8, due to cations being more strongly bound to the soil and not as readily exchangeable. Metals (Cu, Fe, Mn, Ni, and Zn) are very tightly bound to the soil at high pH and are therefore more available at low pH levels than high pH levels. Alkaline and especially sodic conditions cause deficiencies of copper and zinc and also of phosphorus (due to its low solubility). If the alkaline soil has high $CaCO_3$ content, potassium deficiency can occur because this can readily leach. Nitrogen may be deficient as well in alkaline soil due to the generally low organic matter content (Rowell 1994). Acidic pH reduces activity of many soil organisms, in extreme cases resulting in an accumulation of organic matter, reduced mineralization, and low availability of nitrogen, phosphorus, and sulfur (Rowell 1994). "Base" cations (Ca, K, Mg) are more weakly bound to the soil and are prone to leaching at low pH. Thus, in acidic soils, the availability of nutrients such as potassium (K), calcium (Ca), and magnesium (Mg) is reduced, while the availability of potentially toxic elements such as aluminum (Al), iron (Fe), and zinc (Zn) is increased (Haby 1993). Exposure of the organisms to Al and heavy metals may result into either stimulation or no change or inhibition of the enzyme activity, which depends upon the type of the metals used (Giller et al. 1998). Arora et al. (2009) reported that Al was deleterious for the enzymatic activities (nitrate reduction, nitrite reduction, nitrogenase, and uptake hydrogenase) of rhizobia. Cu showed inhibitory effect on growth and enzyme activities of *Bradyrhizobium* strain at all concentrations (Arora et al. 2009). However, in *Sinorhizobium meliloti*, all the tested enzymatic activities increased up to the concentration of 0.1 mM Cu (Arora et al. 2009). Dahlin et al. (1997) observed that there is no distinct threshold for metal toxicity, or for detrimental effects of other inputs, partly because the effects depend on site-specific characteristics such as climate and soil type.

Chin-A-Woeng et al. (1998) observed that at pH 5.7, the in vitro antifungal activity of phenazine-1-carboxamide was ten times higher than phenazine-1-carboxylic acid (PCA). PCA activity was completely abolished under near-neutral conditions. de Souza et al. (2003) proved that the activity of DAPG is more against *Pythium* species at acidic rather than at neutral to alkaline pH, in vitro conditions. Only some studies have examined gene expression, as affected by pH from pure cultures isolated from soil. Soto et al. (2004) described the effects of pH and calcium on *nod* gene expression in *S. meliloti* LPU63 and the acid-tolerant *Rhizobium* sp. LPU83. Bacterial cells were incubated with root exudates of alfalfa plants grown in either a pH of 7 or 5.6 with calcium concentrations of 0.7 or 6 mM. In *Rhizobium* sp. LPU83, the *nodC* gene expression was similar at both pH and calcium levels. However, the *nodC* expression in *S. meliloti* LPU63 was adversely affected at the lower pH and in the presence of low calcium concentrations. Soto et al. (2004) suggested that their findings may be due to low acidity causing the quantity or quality of alfalfa exudates to be suboptimal. An inherent problem in studying soil pH effects is its varied influence on multiple parameters. Experimentally manipulating the pH of soil may result in changes in several factors that are hard to separate (Rousk et al. 2009).

Temperature

Temperature is an important environmental factor regulating soil microbial biomass, activity, and community (Wu et al. 2010). Temperature directly affects the activity of the soil biota by determining the rate of physiological activity such as enzyme activity and indirectly by affecting physicochemical properties such as diffusion and solubility of nutrients, mineral weathering, evaporation rates, and so on. Organisms have a specific range of temperature at which their biological activity operates. So far, most effort has been made to understand how increased soil temperature influences the CO_2 efflux from soil. In comparison, physiology and composition of the microbial community were rarely studied. There is evidence that warming potentially alters decomposer physiology and accordingly the CO_2

efflux from soil (Zogg et al. 1997; Bradford et al. 2008; Balser and Wixon 2009; Allison et al. 2010). The mechanisms behind temperature adaptations of soil microbes could be physiological adaptations of single species (Malcolm et al. 2008) or species shifts within the microbial community. Lipson et al. (2009) showed that microbial winter and summer communities differed in growth kinetics, biomass-specific respiration rates, and respiration rates. Schindlbacher et al. (2011) reported that soil warming did not affect microbial biomass, nor did warming affect the abundances of most microbial groups. Warming significantly enhanced microbial metabolic activity in terms of soil respiration per amount of microbial biomass. To elucidate the effect of warming on the microbial community, Schindlbacher et al. (2011) analyzed soil from the soil warming experiment at Achenkirch, Austria. Soil of a mature spruce forest was warmed by 4 °C during snow-free seasons since 2004. Repeated soil sampling from control and warmed plots was done from 2008 until 2010. They observed that the 4 °C increase in soil temperature during the snow-free season had no influence on microbial community composition and biomass but strongly increased microbial metabolic activity and hence reduced carbon use efficiency.

Changing soil temperature especially warming has been shown to increase the mortality rate of soil microbes (Wu et al. 2010). High soil temperatures in tropical and subtropical areas are a major problem for biological nitrogen fixation of leguminous crops (Michiels et al. 1994). Temperature affects root hair infection, bacteroid differentiation, nodule structure, and the functioning of the legume root nodule (Roughley 1970). Nodule functioning in common beans (*Phaseolus* spp.) is optimal between 25 and 30 °C and is hampered by root temperatures above 30 °C (Piha and Munnus 1987). Piha and Munnus (1987) reported that bean nodules formed at 35 °C were small and had low specific nitrogenase activity. For most rhizobia, the optimum temperature range for growth in culture is 28–31 °C, and many are unable to grow at 37 °C (Graham 1992). However, 90 % of cowpea *Rhizobium* strains obtained from the hot, dry environment of the Sahel Savannah grew well at 40 °C (Eaglesham and Ayanaba 1984). Strain adaptation to high temperature has also been reported by Hartel and Alexander (1984) and Karanja and Wood (1988). The latter found that a high percentage of the strains that persisted at 45 °C lost their infectiveness at high temperature. They attributed these losses in infectiveness to plasmid curing. If nodules are formed at high temperatures, they were ineffective and plants did not accumulate nitrogen in shoots (Hungria and Franco 1993). Heat treatment of *Rhizobium phaseoli* at 35 and 37 °C resulted in mutant strains lacking a plasmid DNA implicated in the synthesis of melanin and is related to the loss of symbiotic properties of these bacteria (Beltra et al. 1988).

There are reports that low temperature near 15 °C caused inhibitory effect on *Bradyrhizobium japonicum*–soybean symbiosis (Antoun and Prevost 2005). It was shown that the presence of plant growth-promoting rhizospheric strains of *Serratia proteamaculans* 1–102 or *Serratia liquefaciens* 2–68 allowed a better plant growth and higher nitrogen fixation and nodule numbers by *Bradyrhizobium* at root zone temperatures of 15 and 25 °C (Zhang et al. 1996, 1997). Pietikäinen et al. (2005) measured the instantaneous total activity (respiration rate), bacterial activity (growth rate as thymidine incorporation rate), and fungal activity (growth rate as acetate-in-ergosterol incorporation rate) in soil at different temperatures (0–45 °C). Fungal and bacterial growth rates had optimum temperatures around 25–30 °C, while at higher temperatures, lower values were found. This decrease was more drastic for fungi than for bacteria, resulting in an increase in the ratio of bacterial to fungal growth rate at higher temperatures. A tendency towards the opposite effect was observed at low temperatures, indicating that fungi were more adapted to low-temperature conditions than bacteria. Fernando et al. (2005) reported that in *P. fluorescens* CHA0, the concentration of DAPG accumulated at 18 °C was twice the concentration at 30 °C. Little research has been conducted investigating temperature as a factor that affects

microbial gene expression in soil. It is known that temperature plays an important role in processes which take place in the soil, including chemical reactions and biological interactions. The soil temperature varies in response to exchange processes that take place primarily at the soil surface. Therefore, it is thought that fluctuations in temperature will have a profound effect on microbial gene expressions within a soil environment. Little is known about the responses of soil microbes coming from different climatic regions to different incubation temperatures. Wu et al. (2010) showed that the majority of soil microorganisms coming from the hot regions are more adapted to high temperature (35 °C) compared to those from the cold area. The effect of incubation temperature on soil microbial community structure was inconclusive. However, genotype experiments are required to be done for the better understanding of fate of inoculant microbes from different climate regions with concerning temperature variation in soils.

Soil Amendment with Agrochemicals

Agriculture is directly dependent upon certain microbiological processes which build and maintain soil fertility. In relation to this, addition of agrochemicals to soil involves certain risks. Even small changes in the chemical structure of compounds can change their biological effects greatly and unpredictably (Niewiadomska and Klama 2005). For example, pesticides used in leguminous crops often show side effects, influencing the symbiotic efficiency and nitrogenase activity (Gaur 1980; Niewiadomska and Klama 2005). Application of lindane to soil at the rate 5 ppm and above inhibits nodulation and growth yield of chickpea, whereas lower concentrations are nontoxic. It was reported that dimethioate and monocrotophos reduced the germination, while carbofuran, formothion, and dimethioate reduced the plumule weight of green gram (Musarrat and Haseeb 2000). Also, treatment with malathion results in the reduced germination of *Vigna mungo*. It is speculated that certain pesticides exhibiting high affinity for plant lectins may interfere with the binding of *Rhizobium* spp. to lectins on the root surface of specific legumes. As a result, the interactions between the legume and *Rhizobium* from the analogous cross-inoculation groups will be inhibited (Musarrat and Haseeb 2000). Niewiadomska and Klama (2005) showed that the nitrogenase activity of *S. meliloti* in the presence of carbendazim and thiram dropped by 93 %, whereas in the presence of imazethapyr by 91 %, compared to the control. In testing procedures for the side effects of pesticides on soil microorganisms, it has long been recognized that effects are more likely to be seen on light-textured soils that are low in organic matter than on heavier soils, and it is therefore recommended to use at least two contrasting soil types for study (Somerville et al. 1987).

In modern agricultural production, herbicide application is a regular practice. Herbicides adsorb on cell surface, affecting ion transport. They affect the metabolism inside the cell by binding to amino and sulfide groups. In the course of these processes, changes take place in the oxidoreduction level of soil, and depending on the chemical composition and dose of herbicide, the microorganism concerned may be killed. Bioaccumulation mitigates the toxic effect of herbicides. Miloševiã et al. (2001) showed that, generally, herbicides tended to reduce the total number of soil microorganisms 7–30 days after application. Miloševiã et al. (1998, 2000) reported that soybean treatment with flumetsulam + trifluralin (Rival), imazethapyr (Pivot), clomazone (Command), and alachlor + linuron (Linuron S-50) reduced the total number of microorganisms in the period of 14 days after application by 15–27 %. After that period, the numbers of microorganisms in the treated variants reached the level of the control variant. The studies have shown that *Azotobacter* is most sensitive to herbicide application (Miloševiã et al. 1995; Miloševiã and Govedarica 2000). Herbicide application also inhibits the symbiotic bacterium *B. japonicum*. Herbicide application reduced nodulation rate, i.e., the number of nodules formed on soybean roots, by 5–21 % (Miloševiã et al. 2000). Studies have also shown that some herbicide applications stimulated the growth and

development of actinomycetes. Dimethenamid and metolachlor applied to sugar beet (Govedarica et al. 2001) and prometryn applied to soybean and sunflower (Miloševiã and Govedarica 2001) increased the number of actinomycetes. Certain microbial species may decrease or increase the toxic action of herbicides. Most soil microorganisms are capable of decomposing herbicides, using them most frequently as sources of biogenous elements. The hormone herbicide 2,4-D is rapidly decomposed in the soil, as much as 3.4×10^6 mol in a single day. Various microorganisms decompose 2,4-D: *Mycoplana*, *Corynebacterium*, *Achromobacter*, *Rhizobium*, *Arthrobacter*, *Flavobacterium*, and some actinomycetes (Lynch 1983). According to Kosinkievicz (1984), the phenolic compounds produced by *Pseudomonas acidovorans* may increase or decrease the phytotoxicity of lenacil (Venzar), the mode of action depending on herbicide concentration. A detailed description of impacts of various pesticides on soil microbial communities is summarized in Table 19.1.

Farmers commonly report that the long-term use of synthetic fertilizers, especially anhydrous ammonia, leads to soil compaction and poor tilth (Francis et al. 1990). When bacteria increase and organic matter decreases, aggregation naturally declines because there is no more glue being produced to stick the soil particles together. Anhydrous ammonia used as fertilizer contains approximately 82 % nitrogen and is applied subsurface as a gas. Anhydrous ammonia speeds the decomposition of organic matter in the soil, leaving the soil to be more compact. The addition of anhydrous ammonia contributes acidity to the soil, requiring 148 lb of lime to neutralize 100 lb of anhydrous ammonia or 1.8 lb of lime for every pound of nitrogen contained in the anhydrous (Tisdale et al. 1985; Francis et al. 1990). Anhydrous ammonia initially kills many soil microorganisms in the application zone. Bacteria and actinomycetes recover within 1–2 weeks to levels higher than those prior to treatment (Francis et al. 1990). Soil fungi, however, may take 7 weeks to recover. During the recovery time, the bacteria are stimulated to grow and decompose more organic matter due to the high soil nitrogen content. This is why their numbers increase after anhydrous ammonia applications. Sodium nitrate, also known as Chilean nitrate or nitrate of soda, is a high salt fertilizer. The concern is that excessive sodium acts as a dispersant of soil particles, degrading aggregation.

Organophosphates are another group of fertilizers commonly used in agricultural lands, responsible for the decrease in N-mineralization process (Sato 1983). Mycorrhizal fungi have been consistently reported as being decreased by P fertilizer, but the extent to which this occurs may be dependent on species of fungus involved and level of plant available P in soil (Seymour 2002). The appliance of biopesticides in these chemical fertilizer-supported soils needs understanding of proper application timing and field trials. Despite the numerous efforts aimed at understanding the effects of agrochemicals on the soil ecosystem, it is difficult to comprehend the role of agrochemicals in perturbing soil environment due to divergent research findings reported in the literature. Generally, long-term application of agrochemicals can disturb biochemical equilibrium which can reduce soil fertility and productivity. Understanding the mechanisms underlying molecular responses in microbes in response to agrochemical application could be helpful in elucidating the risk assessment of agrochemical contaminations and its consequent adverse impacts on soil microbial diversity, enzymatic activities, and biochemical reactions.

Effect of Soil Biotic Factors

Soil Indigenous Microbes

In order to have a beneficial effect on a target plant, PGPMs are introduced in large number with the aim of having good root colonization, a prerequisite for the successful use of PGPM. Microbial inoculant colonization is affected by interactions with other soil microorganisms. Chanway and Holl (1992) provided anecdotal evidence to support the view that changes in soil microbial composition cause variability of plant growth responses. Little work has been done to investigate the relationship between PGPM efficacy and changes in the soil microbial

Table 19.1 Effect of agrochemicals on soil microorganisms

Pesticides	Effect on soil microbes	References
HCH, phorate, carbofuran	Increased soil microbial population including N_2-fixing bacteria and P-solubilizing microorganisms	Das and Mukherjee (2000)
Atrazine, isoproturon, metribuzin, sulfosulfuron	Adversely affected *Bradyrhizobium* sp.	Khan et al. (2006)
Mefenoxam, metalaxyl	Inhibited N-fixing bacteria	Monkiedje et al. (2002)
Carbofuran, ethion	Adversely affected soil microorganisms	Kalam and Mukherjee (2001)
Hexaconazole	Reduction in nitrogenase activity of *Anabaena doliolum*	Hammouda (1999)
Phorate	Decreased total number of bacteria and N_2-fixing bacteria	Gonzalez-Lopez et al. (1993)
Malathion	Increased number of denitrifying bacteria; nitrifying and fungal populations were not affected	Gonzalez- Lopez et al. (1993)
Captan	Decreased viable counts of rhizobia; fungi, nitrifying and N2-fixing bacteria significantly decreased, while denitrifying bacteria increased; nitrogenase activity decreased in soils under aerobic conditions	Kyei-Boahen et al. (2001) Martinez-Toledo et al. (1998)
DDT, 2,4-D	Inhibited the growth of *Rhizobium* sp.; inhibited *nod* expression by 32–90 % by disrupting plant–*Rhizobium* signaling	Fabra et al. (1997) Mclachlan (2001)
Isoproturon	Biodegradation of isoproturon-favored bacterial growth while suppressed actinomycetes and fungi	Nowak et al. (2004)
Butachlor	Stimulated anaerobic fermentative and sulfate-reducing bacteria while inhibited acetogenic bacteria in paddy soil	Min et al. (2001)
Ridomil gold plus copper	Increased N- and organic P-mineralization, ammonification	Demanou et al. (2004)
Carbendazim, imazetapir, thiram	Inhibited nodulation; reduced nitrogenase activity in rhizobia	Niewiadomska (2004)
Pentachlorophenol	Reduction in nitrification rates	Colores and Schmidt (2005)
Lindane	Nitrification and phosphate solubilization decreased	Ogunseitan and Odeyemi (1985)
Organophosphates	Denitrifying bacteria decreased thus N-mineralization decreased; decreased root colonization and/or spore number of mycorrhiza	Sato (1983) Seymour (2002)
N- fertilizers	Mycorrhizal fungi spore number and root colonization decreased; adversely affect soil bacteria	Jensen and Jakobsen (1980) Francis et al. (1990)

communities in which PGPMs function. One type is mutualism – interactions beneficial to both parties, such as those involving mycorrhizae, symbiotic nitrogen fixers, and many rhizosphere microbes. The second type of biotic interactions is competition and antagonism for limited food and habitat (Chapin et al. 2002).

Several reports from the Himalayan region have shown that the effects of seed inoculation on plant growth may be due to stimulation of already existing native microbial communities of the rhizosphere/rhizoplane. Pandey et al. (1998) have observed a two- to five fold increase in population of actinomycetes (a group known to fix nitrogen), following

inoculation of maize with *Azotobacter chroococcum* and *Azospirillum brasilense* at the Mamlay watershed in Sikkim. Bacterial inoculation of tea rhizosphere resulted in the stimulation of the native bacteria, actinomycetes, and a group of bacteria capable of growing on N-free medium while suppressing the rhizospheric fungal population (Trivedi et al. 2005). Kumar et al. (2007) have also attributed the positive effect of bacterial inoculation on growth of maize through the stimulation of native microflora. However, Robleto et al. (1998) showed that a field release of a *Rhizobium etli* strain containing genes encoding trifolitoxin (an antibiotic peptide active against members of a specific group of α-proteobacteria that enhances the ability to compete trifolitoxin-sensitive strains) strongly reduces the diversity of trifolitoxin-sensitive members of α-proteobacteria in bean rhizosphere as shown by ribosomal intergenic spacer analysis (RISA), with little apparent effect on most microbes.

Soil is generally poor in nutrients and energy sources (estimated to be only about 5 % of its solid material), when compared with the conditions used for microbial growth under laboratory conditions (Ashman and Puri 2002; Nannipieri et al. 2003). Microorganisms are thus constantly competing for nutrient sources (Bridges 1998), mainly carbohydrates, nitrogen, phosphorus, and ions such as iron (Fe^{3+}) and calcium (Ca^{2+}) (Miller and Donahue 1995; Stengel and Gelin 2003). Various studies have shown that the ecologically competent bacteria can effectively establish in a plant environment as compared to nonnative strains. Further, the effect of a particular strain to promote plant growth is also dependent on its interaction with other beneficial microorganisms present in the vicinity of target plants (Trivedi et al. 2012). Latour et al. (2003) described a strategy to study traits involved in the rhizosphere competence. First, the diversity of indigenous populations associated with plant roots was compared with that of the uncultivated soils in order to identify traits discriminating between the two populations. Comparing a wild-type strain to mutants affected in the corresponding phenotypes allowed the determination of the involvement of the traits involved in rhizosphere competence. Finally, traits shared by populations adapted to the rhizosphere were identified by comparing the metabolism and the competitiveness in the rhizosphere of a collection of bacterial strains. The results obtained indicated that rhizosphere-competent pseudomonads are particularly efficient in using pyoverdine-mediated iron uptake system and in reducing nitrogen oxides (Latour et al. 2003).

The agonistic and antagonistic effects of soil microbes through various interactions of bacteria, fungi, and *Actinomycetes* on *Rhizobium* have profoundly influenced sustainable annual harvests (Parmar and Dufresne 2011). Nine *Streptomyces* spp. were isolated from the nodule surface of black gram which were found to inhibit fast- and slow-growing strains of cowpea and soybean rhizobia. The fast-growing strain of *Rhizobium* (both cowpea miscellany and soybean) was more sensitive to antibiosis as compared to slow-growing stains (Jayaraman et al. 1985; Pugashetti et al. 1992). Another study observed that 90 % of the *Actinomycetes* sp. isolated from soil obtained from field plots was antagonistic to *B. japonicum* (Pugashetti et al. 1992). Conn and Franco (2004) found a significant reduction in indigenous actinobacterial endophytes upon inoculation of soil with a commercial multi-organism product, compared with no change in diversity after inoculation with a single species. Most inoculants often rely on application of a single strain which might partially account for the recorded inconsistencies in the field. However, for utilization of multi-organism products, thorough investigation is necessary as the combination of inoculants will not necessarily produce an additive or synergic effect, but rather a competitive process, and, hence, growth enhancement could be reduced or eventually disappear (Trabelsi and Mhamdi 2013). Many researchers have given conclusion that in the rhizosphere, PGPMs (e.g., *Pseudomonas* and *Bacillus*) are involved in interactions with root-nodulating bacteria. Interactions of these rhizobacteria with *Rhizobium* may be antagonistic or synergistic (Guaiquil and Luigi 1992; Capper and Higgins 1993; Parmar and Dadarwal 1997).

It is now clear that the development of endo- or ectomycorrhizae cause rhizosphere microbial changes which can result in interactions among rhizosphere microbes (Bianciotto and Bonfante

Table 19.2 Signal molecules of the quorum-sensing systems identified from microorganisms

Autoinducers	Producing species	Phenotype(s) regulated	References
Acylated homoserine	Many Gram-negative bacteria	Bioluminescence, plasmid conjugal transfer, biofilm formation, motility, antibiotic biosynthesis, and production of virulence factors	Eberhard et al. (1981), Ryan and Dow (2008)
Quinolones	*Pseudomonas aeruginosa*	Elastase, rhamnolipid, pyocyanin, production, and biofilm formation	De'ziel et al. (2004), Diggle et al. (2006)
Autoinducer-2	Many Gram-negative, Gram-positive bacteria, and oomycetes	Bioluminescence, ABC transporters	Xavier and Bassler (2003), Vendeville et al. (2005), Kong et al. (2010)
Bradyoxetin	*Bradyrhizobium japonicum*	Nodulation	Loh et al. (2002)
Butyrolactone	*Streptomyces*	Morphological differentiation and secondary metabolite production	Chater and Horinouchi (2003)
Cyclic dipeptides (CDPs)	*Pseudomonas fluorescens*, *P. alcaligenes*	Influence N-AHL-dependent quorum sensing	Holden et al. (1999), Degrassi et al. (2002)
Fatty acid derivatives	*Ralstonia solanacearum*	Prokaryotic differentiation	Flavier et al. (1997)
Diffusible signal factor	*Xanthomonas campestris*	Endoglucanase production	Wang et al. (2004)
Indole and derivatives	Many Gram-negative and Gram-positive bacteria	Virulence, biofilm formation, drug resistance, sporulation	Kim et al. (2011)
Short peptides	Gram-positive bacteria	Genetic competence	Okada et al. (2005)

2002). Linderman and Paulitz (1990) reviewed the interactions between mycorrhizae and groups of bacteria such as nitrogen-fixing bacteria, PGPR including phosphate-solubilizing bacteria, and biocontrol agents. Bacteria associated to mycorrhizal fungi adhere to fungal spores and hyphal structures and thus spread to the rhizosphere (Bianciotto and Bonfante 2002). Recently, Bianciotto et al. (2004) observed strong evidence of a vertical transmission of endobacteria through the arbuscular mycorrhiza (AM) fungus vegetative generation. In a study using the nitrogen-fixer *A. chroococcum* and *P. fluorescens*, the chemotaxis of these two PGPRs towards the roots of mycorrhizal tomato plants (*Glomus fasciculatum*) was an important step of communication for root colonization (Sood 2003). It was found that *G. fasciculatum* alters the characteristics of root exudates which are chemoattractants specific for each PGPR, amino acids for *P. fluorescens*, and sugars for *A. chroococcum*. Silveira et al. (1995) showed in experiments with beans (*Phaseolus vulgaris*) that bacterial culture of fluorescent *Pseudomonas* co-inoculated with *Glomus etunicatum* increased root growth, nodulation, and N and P uptake. However, the interactions of PGPR with AM fungi are often contradictory and probably depend on the bacterium, the plant species, and the environmental factors.

Complex biotic factors such as plant species, plant age, host cultivar, and infection with the plant pathogen can significantly alter the expression of the gene *phlA*25 for DAPG production in fluorescent pseudomonads (Dwivedi and Johri 2003). Bacteria can perceive extracellular conditions using numerous sensor/kinase signal transduction pathways (Gross et al. 1989). Quorum sensing enables bacteria to regulate their gene expression in a population density-dependent way and adjust their physiology according to their environmental conditions and coordinate the behavior of the entire cell population. Many bacterial functions related to pathogenicity or symbiotic interactions with plants or animals are regulated by quorum-sensing systems. To date, several classes of extracellular signal molecule have been described in microorganisms (Table 19.2). Most QS signals are either small (1,000 Da) organic molecules or peptides with 5–20 amino acids (Chhabra et al. 2005; Williams

et al. 2007). Gram-negative bacteria, for example, employ N-acylhomoserine lactones (AHLs), 2-alkyl-4(1H)-quinolones (AQs), long-chain fatty acids, and fatty acid methyl esters as well as autoinducer-2 (AI-2), a collective term for a group of interconvertible furanones derived from dihydroxypentanedione (DPD). AI-2 is also produced by some Gram-positive bacteria, although generally these organisms prefer linear, modified, or cyclic peptides such as the autoinducing peptides (AIPs) made by the staphylococci (Xavier and Bassler 2003). AI-2 production has also been reported in fungi (Kong et al. 2010). Signal molecules belonging to further structural classes such as indole and its derivatives have also been described (Kim et al. 2011). Since two or more bacterial species can use identical or the same class of signal molecules for interspecies communication, cross talk may occur between different signaling systems. Microcolonies on the root surface can indeed consist of more than one population or strain (Bloemberg et al. 2000; Dekkers et al. 2000). Khare and Arora (2011) reported that pyocyanin, an antibiotic from fluorescent pseudomonads at lower concentrations, acts as signaling molecules and modulate gene expression. The application of pyocyanin-producing pseudomonads together with rhizobia contributes to the enhancement of nodulation ability.

Phenotypic and genotypic approaches are now available to characterize the rhizobacterial community structure. Methods that rely on the ability to culture microorganisms include standard plating methods on selective media, community level physiological profiles (CLPP) using the BIOLOG system (Garland 1996), phospholipid fatty acid (PLFA; Tunlid and White 1992), and fatty acid methyl ester (FAME profiling; Germida et al. 1998). Culture-independent molecular techniques are based on direct extraction of DNA from soil and 16S rRNA gene sequence analysis, bacterial artificial chromosome, or expression cloning systems. These provide new insight into the diversity of rhizosphere microbial communities, the heterogeneity of the root environment, and the importance of environmental and biological factors in determining community structure (Smalla et al. 2001). Recently, microarray technique was adapted to elucidate the structure (phylogenetic oligonucleotide microarrays) and functions (functional gene arrays, FGA) of microbial community. Pyrosequencing has broken the barrier of sequence limitations in the study of bacterial diversity. The ability to generate megabases of sequences in a few hours allows intense exploration of species in any environmental sample (Edwards et al. 2006). These approaches can also be used to determine the impact of inoculation of plant growth-promoting rhizobacteria on the rhizosphere community.

Soil Fauna

Different types of soil microfauna (protozoa and free-living nematodes), mesofauna (collembola and mites), and macrofauna (earthworms and termites) play a significant role in soil biological processes (Coleman et al. 2004). Grazers and predators release plant-available nutrients as they consume microbes. Often, organisms specialize in one type of prey, such as either bacteria or fungi. Certain collembolans (springtails) even specialize on specific species of fungi. Other organisms are generalists and will feed on any microbial species they encounter (Hol et al. 2013). Several investigations from freshwater (Pernthaler et al. 1997; Jürgens et al. 1999; Posch et al. 1999) but also from soil systems show that protozoa do not indiscriminately ingest bacteria, but selectively feed on certain bacterial strains (Griffiths et al. 1999; Rønn et al. 2002; Rosenberg 2008). Ciliates and flagellates have distinct and constant cell shapes and feed on free-swimming soil bacteria, whereas amoebae have more plastic bodies that continually change shape and ingest organisms attached to soil particles (Hausmann and Hülsmann 1996; Clarholm et al. 2006). Bonkowski and Brandt (2002) demonstrated that grazing of soil protozoa on bacteria shifts the composition of the microbial community towards auxin-producing bacteria. Generally, feeding by flagellates and ciliates is size selective (Jürgens and Güde 1994; Jürgens et al. 1999; Hahn and Höfle 2001). Medium-size bacterial cells are most susceptible to predation by flagellates and ciliates, whereas

smaller cells and large filamentous forms may be partly resistant to grazing (Jürgens and Güde 1994; Hahn and Höfle 2001). Hence, grazing may lead to a bidirectional shift in the relative distribution of bacterial size classes, with increases in the relative abundance of large filaments and small cells (Hahn and Höfle 2001).

A study using DNA fingerprinting (denaturing gradient gel electrophoresis [DGGE] analysis of PCR-amplified 16S rRNA gene sequences), CLPP, and phospholipid fatty acid analysis demonstrated that protozoan grazing changed the compositions of the microbial communities in soil microcosms towards Gram-positive bacteria (Griffiths et al. 1999). DGGE of PCR amplifications of 16S rRNA gene fragments then excising and sequencing of bands from the DGGE gels indicated that high G+C Gram-positive bacteria closely related to *Arthrobacter* spp. were favored by grazing, whereas the excised bands that decreased in intensity were related to Gram-negative bacteria (Rønn et al. 2002). As reported by Rønn et al. (2002), five different protozoan isolates (*Acanthamoeba* sp., two species of *Cercomonas*, *Thaumatomonas* sp., and *Spumella* sp.) had different effects on the bacterial communities. This suggests that the composition of protozoan communities is important for the effect of protozoan grazing on bacterial communities.

Among predatory prokaryotes, *Bdellovibrionaceae* species (small deltaproteobacteria) prey on other Gram-negative cells, using flagella to swim rapidly until they collide with a prey cell (Morgan et al. 2010). After collision, the predator cells then enter the periplasmic space of the prey cell, consume the host cell from within, elongate, and divide into new cells that are released upon host cell lysis (Lambert et al. 2006). Predation by myxobacteria, which are also δ proteobacteria but constitute a monophyletic clade divergent from the *Bdellovibrionaceae*, was also reported by several workers (Morgan et al. 2010). Myxobacteria are found in most terrestrial soils and in many aquatic environments as well (Dawid 2000; Velicer and Vos 2009) and exhibit predation on both bacteria and fungi (Morgan et al. 2010). Myxobacteria use gliding motility (Spormann 1999) to search the soil matrix for prey and produce a wide range of antibiotics and lytic compounds that kill and decompose prey cells and break down complex polymers, thereby releasing substrates for growth (Sudo and Dworkin 1972). There is evidence that *Myxococcus xanthus* employs chemotaxis-like genes in its attack on prey cells (Berleman et al. 2008) and that predation is stimulated by close contact with prey cells (McBride and Zusman 1996).

There are reports on root-knot nematodes as potential inhibitors of nodulation in legumes, particularly mung bean, chickpea, cowpea, and green gram. The presence of sugars such as *N*-acetylglucosamine, galactose, *N-acetylgalactosamine*, and mannose and/or glucose on the cuticle surface of plant-parasitic nematodes may play an important role in the interaction between nematodes and their hosts (Musarrat and Haseeb 2000). It has been demonstrated that the binding of rhizobia to nematode-free roots was inhibited only after pretreatment with certain sugars (Huang et al. 1984). Studies on the interference of nematodes with soybean lectin metabolism showed the reduced binding of rhizobia to *Heterodera glycines*-infected soybean roots, suppressing the nodule formation (Huang et al. 1984). Selective grazing can change bacterial competition (Pedersen et al. 2009). Bacterivores avoid *P. fluorescens* due to the secondary metabolite production by them. This can improve the competitive advantage of *P. fluorescens* over other bacteria (Jousset et al. 2008; Jousset 2012). *Pseudomonas* can produce hydrogen cyanide and thus repel bacterivorous nematodes. Also DAPG, an antibiotic compound produced by *P. fluorescens*, acts as nematicide (Neidig et al. 2011).

Decomposer soil invertebrates also affect the distribution of microorganisms among soil particle-size fractions, their activity, and, therefore, mobilization and stabilization processes of organic matter by mixing organic and mineral soil components during the gut passage (Scullion and Malik 2000; Marhan and Scheu 2006). A large number of soil invertebrates are involved in these processes, but due to their large size and dominance in numbers and biomass in many terrestrial ecosystems, earthworms are the most important drivers (Beare et al. 1995; Bardgett

2005). Fluorescence microscopy, genetic fingerprinting (T-RFLP analysis), and phospholipid fatty analysis suggests that the general structure of microbial communities remains little affected by the gut passage through earthworms (Egert et al. 2004; Marhan et al. 2007). However, by feeding on both organic matter-rich patches and mineral soil and intimately mixing them during the gut passage, the structure and activity of microbial communities are strongly affected (Devliegher and Vertraete 1995) and alter the distribution of microorganisms associated with soil macro- and micro-aggregates (Mummey et al. 2006). Further, despite the structure of microbial communities may be little affected, their activity may change markedly, e.g., it has been documented recently that the activity of denitrifiers is strongly increased in earthworm guts and fresh cast materials (Drake and Horn 2006; Horn et al. 2006). Macrofauna have been shown to reduce pathogen inocula (*Rhizoctonia solani*, *Fusarium* spp.), disperse beneficial organisms (e.g., *Rhizobium*, biocontrol bacteria), and release nutrients from crop residues (Stephens et al. 1993; Doube et al. 1994; Baker 2007).

Conclusion

On the application of bioformulation in soil, its immediate response varies considerably depending on plant species, soil type, soil biotic factors, inoculant density, and environmental conditions. This results in a progressive decline in the inoculant density which fails to elicit intended plant response. Soil texture is an important factor that influences distribution of minerals, organic matter retention, microbial biomass, and other soil properties (Scott and Robert 2006). Accordingly, pore space distribution plays a major impact on the abundance of bacteria and fungi and might be responsible for higher rates of carbon mineralization (Jensen et al. 1996; Raiesi 2006). The soils in good physical condition have better aeration and moisture content which is essential for optimum microbial activity. Fluctuations in nutrient availability, temperature, and water status greatly influence the growth, survival, and metabolic activity of soil microorganisms and plants and may quickly diminish the population of any microbe introduced into soil (Van Veen et al. 1997).

The soil being a heterogeneous, unpredictable environment, the inoculated microbe finds it often difficult to establish a niche for survival among the competitors and predators. Microorganisms interact with each other giving rise to antagonistic or symbiotic interactions. The association existing between one organism and another whether of symbiotic or antagonistic nature influences the population and activity of soil microbes to a great extent. On the other hand, the activities of some of the microorganisms are beneficial to each other. For instance, organic acids liberated by fungi, increase in oxygen by the activity of algae, and change in soil reaction favor the activity of bacteria and other organisms in soil. The predatory habit of protozoa and some myxobacteria which feed on other bacteria may also suppress or eliminate certain inoculants (Chapin et al. 2002). The interactions of microbial inoculants with soil abiotic and biotic (indigenous soil organisms) factors are likely to be complex, and a better mechanistic understanding is necessary to predict short- and long-term effects.

After being introduced into the soil, the inoculants have to compete with native soil microorganisms for the nutrient and habitable niche and have to survive against grazing protozoa. The inoculants can be amended with nutrients to improve the short-term survival of cells upon inoculation, which is essential to the success of any bioformulation (Trivedi et al. 2012). Such carrier materials that offer the available nutrient and/or habitable micropore to the inoculants will be desirable for the development of future bioformulations. In this sense, materials with microporous structure, such as soil aggregate and charcoal, will be good carriers for soil inoculants (Malus'a et al. 2012). Further, to make effective utilization of microbial inoculants, accurate and reliable methods for monitoring the fate of introduced PGPM in the rhizosphere/rhizoplane are required to enhance their efficacy under field conditions. Proteomics can be exploited as a

powerful tool to understand the complex patterns of expression of genomes with respect to different environmental niches in which the bacteria adapt and survive. Metabolomics (the quantitative complement of all low-molecular-weight molecules present in the cells in a particular physiological or developmental state) techniques have a huge potential to be applied for strategies aimed at improving competitive abilities of biofertilization and biocontrol strains (Narasimhan et al. 2003; Trivedi et al. 2012). The chance of obtaining a successful inoculant in the end will be greatly enhanced when ecological principles are applied throughout the procedure of development of microbial inoculants. Only then, the major goal of inoculant formulation to provide more suitable microenvironment for survival of introduced PGPR in soil will be fulfilled.

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